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(54) Title: MAMMALIAN CELL CYCLE PROTEIN (57) Abstract A novel mammalian cell cycle protein, p55CDC, DNA sequences encoding p55CDC, and a method for producing the protein are described. Also described are methods for detecting p55CDC and methods for modulating cell division by compounds which control the level or activity of p55CDC or p55CDC-associated protein complexes.		

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MAMMALIAN CELL CYCLE PROTEIN

5 The invention relates to a mammalian cell
cycle protein, p55CDC, DNA sequences encoding same,
antibodies specific for the protein, a method for
producing the protein and methods for modulating cell
division by controlling the levels or activity of p55CDC
or p55CDC-associated protein complexes.

10

BACKGROUND OF THE INVENTION

15 The eukaryotic cell cycle has a growth phase
and a reproductive phase, the latter composed of the
chromosome cycle and the centrosome cycle which
intersect in the establishment of the mitotic apparatus
(for review, see 47). The profound morphologic changes
which result in mitosis are accompanied by a cascade of
phosphorylation and dephosphorylation events. In
20 mammalian cells, different complexes of kinases and
their associated regulatory proteins control progression
through discrete steps of the cell cycle (for review,
see 60,67). While all eukaryotic cells use similar
mechanisms to regulate progression through the stages of
25 the cell cycle, it is clear that unique combinations of
regulatory cyclins, kinases and phosphatases are
responsible for cell- and organism-specific patterns of
cell division (18,51,52).

30

A variety of kinases have been identified
which control the crucial transitions through the cell
cycle. The most well characterized is the p34cdc2
protein, which has been identified in all eukaryotic

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cells which have been examined (3, 16, 20, 28, 40, 41, 42, 59, 76). In addition, many other kinases which have homology to p34cdc2 and, like p34cdc2 fluctuate in activity during the cell cycle, have been described (48,60). Other types of kinases have also been shown to vary in activity at different stages of the cell cycle, and have been proposed to play a role in control of cell division, although they share little or no homology with p34cdc2. These include the MAP kinases, and the MEK kinases which regulate MAP kinase activity (for review, see 11). In addition, a novel kinase has been identified in the fungus *Aspergillus nidulans*, the NIMA kinase, which is required to initiate mitosis (53-55). A mammalian kinase, Nek1, which has homology to the NIMA kinase, has been found in mouse, where it is expressed at high levels in gonadal tissues and may be required for meiosis (43).

As mentioned above, the activity of many of these kinases is regulated by their association with one or more cyclins. The cyclins are homologous with one another within a conserved region termed the cyclin box (44). The fluctuations in activity of the cyclin dependent kinases during the cell cycle result from differential association with newly synthesized cyclins, which are then degraded at specific transition points in the cell cycle. However, not all cyclins demonstrate the same degree of fluctuation during the cell cycle; for example, levels of the D type cyclins do not oscillate as dramatically during the cell cycle as the A and B type cyclins. In addition, a recently described cyclin, the mcs2 cyclin of *S. pombe*, shows no variation in level during the cell cycle, nor does the novel kinase activity associated with the mcs2 cyclin oscillate (49).

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Experiments in yeast have defined a number of other cell division cycle (Cdc) proteins which are also crucial for the orderly progression of the cell cycle, although the functions of many of these proteins have not been precisely defined (34). Two of these proteins, the products of the *CDC20* and *CDC4* genes, have been proposed to be elements of the mitotic spindle or segregational apparatus (32). The *cdc20* temperature sensitive mutants arrest in mitosis at the non-permissive temperature, after the formation of a complete short spindle and nuclear migration to the neck between the mother cell and a large bud (6). It has been proposed that the Cdc20 protein is directly required for chromosomal movement (56). In addition, the Cdc20 protein is required for modulation of microtubule structure, either by promoting microtubule disassembly (1,65) or by altering the surface of the microtubules, and is also required for microtubule-dependent processes other than mitosis (65).

20

The *CDC4* gene of *S. cerevisiae* (33), is essential for the initiation of DNA synthesis. Cells carrying a conditional-lethal, temperature-sensitive mutation in *cdc4* arrest division at the non-permissive temperature, and the cells have a termination phenotype of multiple buds, a single nucleus, and duplicated spindle pole bodies connected by a bridge structure (6). *CDC4* also appears to be required for karyogamy and sporulation (21,68,71). While the mechanism of action of the Cdc4 protein is still unknown, subcellular localization studies in yeast have demonstrated that it is associated with the nucleoskeleton (7). The appearance of the duplicated spindle pole bodies has been proposed to indicate that the *CDC4* gene product is required for separation of the bodies and formation of the completed spindle (6,75). It has recently been

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demonstrated that removal of the centrosome (the equivalent of the spindle pole body in higher eukaryotes) from mammalian cells uncouples the growth cycle from the reproductive cycle, indicating that cell division requires the presence of centrosomes to establish the bipolar mitotic spindle (45).

It is an object of the present invention to identify one or more proteins involved in regulation of the cell cycle, wherein said proteins may be targets for compounds which modulate the cell cycle. A novel protein, termed p55CDC has been identified. mRNA encoding p55CDC was ubiquitously present in all cell lines examined, as well as in embryonic tissue, placenta and adult hematopoietic tissues, but was not detected in cells induced to differentiate and cease cell division. The deduced amino acid sequence of human p55CDC demonstrates regions of homology with the *S. cerevisiae* Cdc20 and Cdc4 proteins within the G β -repeats found in the carboxy terminal half of these three proteins. Expression of p55CDC appears to be crucial for cell division in mammalian cells. p55CDC is phosphorylated in cycling cells. Immune complexes precipitated by a polyclonal antiserum to p55CDC have a kinase activity which fluctuates during the cell cycle, although p55CDC itself does not appear to be an endogenous substrate of the kinase activity.

30

SUMMARY OF THE INVENTION

The invention relates to a novel mammalian protein, p55CDC, which is essential for cell division. It has been found that p55CDC is expressed in actively proliferating cells while expression is not detected in slowly dividing or quiescent cells. Transfection of

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antisense p55CDC cDNA into CHO cells resulted in the isolation of only those cells having a compensatory increase in p55CDC mRNA having the sense orientation.

DNA sequences encoding biologically active p55CDC are also provided by the invention. DNA sequences include rat (SEQ. ID NO: 1) and human (SEQ. ID NO: 3) p55CDC and DNA hybridizing to rat or human p55CDC, or to a fragment thereof, wherein the hybridizing DNA encodes biologically active p55CDC. Also provided for are vectors containing p55CDC DNA sequences and host cells transformed or transfected with said vectors. A method of producing a p55CDC polypeptide comprising culturing transformed or transfected host cells such that p55CDC is expressed is also included

p55CDC polypeptides of the invention will preferably form a complex with one or more host proteins such that the complex has cell-cycle dependent kinase activity. The kinase activity of p55CDC complexes will fluctuate during the cell cycle.

A method for modulating cell division is also encompassed by the invention, wherein the method comprises introducing into a cell (e.g. a tumor cell) a compound which modulates the kinase activity of p55CDC complexes. Modulation of p55CDC associated kinase activity may involve an increase or decrease in activity at certain periods during the cell cycle which in turn may lead to alterations in timing or specificity of p55CDC-associated kinase activity. In a preferred embodiment, cell division is inhibited by exposure to compounds which interfere with p55CDC complex formation.

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DESCRIPTION OF THE FIGURES

Fig. 1. Northern Analysis of p55CDC.

(A) Total RNA (30 μ g) from a variety of rat
5 tissues at different developmental stages was probed
with a rat genomic 0.26 kb *Pst* I fragment.

B) PolyA⁺ RNA (2.5 μ g) from human tissues was
probed with a [³²P] labelled p55CDC cDNA from rat.

(C) PolyA⁺ RNA (2.5 μ g) from human
10 hematopoietic cell lines was analyzed with the same
probe as in Fig. 1B. The signal obtained with a rat
actin cDNA probe is shown for comparison.

(D) Total RNA 30 μ g) prepared from cell lines
that were induced to differentiate as described in
15 Experimental Procedures and from control cells was
probed with the same probe as in Fig. 1B. The ethidium
bromide stain for 28SRNA is shown for comparison. All
details for RNA isolation and Northern blot
hybridization are described in Experimental Procedures.

20

Fig. 2 Rat and Human p55CDC DNA sequence.

The compiled sequence from two rat cDNA clones
is shown. The open reading frame of the human cDNA is
shown only where it differs from the rat sequence.

25 Nucleotide base pair numbers are shown to the left and
amino acids, deduced from the nucleotides, are numbered
at the right. Two in frame stop codons upstream of the
initiation methionine are underlined and a
polyadenylation signal downstream of the stop codon is
30 boxed.

Fig. 3. p55CDC has seven G β -repeats and shows
homology to the *S. cerevisiae* Cdc20 and Cdc4 proteins.

(A) The alignment of the seven rat p55CDC
35 repeats was manually constructed following pairwise
comparisons using the GCG BESTFIT program. Gaps were

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introduced to obtain optimal alignment and are represented by spaces. Identical or highly conserved residues which occur at a frequency of 4 times or greater are shown as white on black. Highly conservative substitutions are defined as Ile, Leu or Val, Ser or Thr, and Ala or Gly.

(B) Alignment of the G β -repeats of human p55CDC with the Cdc20 and Cdc4 repeats was obtained using the GCG BESTFIT program followed by visual optimisation. Gaps were introduced to obtain optimal alignment and are represented by spaces. Identical residues are shown as white on black and the highly conserved residues are boxed. Highly conservative substitutions are defined as Ile, Leu or Val, Ser or Thr, Ala or Gly, Tyr or Phe, Asp or Glu and Arg, Lys or His.

Fig. 4. Southern Analysis of Genomic DNA from various species.

Genomic DNA (10 μ g) from several species was digested with *Hind* III and separated on 1% agarose gels. The filter was probed with rat p55CDC cDNA under medium stringency conditions as defined in Materials and Methods.

Fig. 5. Overexpression of p55CDC cDNA in the sense or antisense orientation in CHO cells results in alteration of growth profiles.

(A) CHOd⁻ cells were transfected with (Δ) PMT, (O) PMTp55s or (\square) PMTp55as DNA and amplified as described in Materials and Methods. Cells were plated at a starting density of 0.5×10^6 cells/60mm dish and counted at the times shown. Arrows indicate the days when media was changed. Each point represents the mean of duplicate counts from parallel cultures which usually varied from 2-14% from the plotted mean.

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(B) Flow cytometry analysis of fixed and propidium iodide stained PMTp55s(—) and PMTp55as (--) cells was performed as described in Materials and Methods.

5

Fig. 6. Immune complexes detected by antibodies against p55CDC.

(A) Cell lysates from ³⁵S-labelled cells in log phase (250 µg on lanes 1,2,3,6,7,8,11,12,13, or 500 µg on lanes 4,5,9,10, and 14) were immunoprecipitated with various antibodies. Immune complexes obtained with 10 µl p34cdc2 MAb (lanes 1, 6 and 11), p55CDC competed antiserum (8.4 µg/lane 2,4,7,9,12) and affinity purified p55CDC antiserum (1 µg/lane 3,5,8,10,13,14) were analyzed on 10% SDS-PAGE gels. The dried gel was exposed for autoradiography for 21 hours.

(B) Cell lysates from ³⁵S-labelled cells in stationary phase (250 µg on lanes 1, 2, 3, 6, 7, 8, 11, 12, 13, or 500 µg on lanes 4,5,9,10,14,15) were immunoprecipitated with various antibodies. Immune complexes obtained with 10 µl p34cdc2 MAb (lanes 1,6,11), p55CDC competed antiserum (8.4 µg/lane 2,4,7,9,12,14) or affinity purified p55CDC antibody (1 µg/lane 3,5,8,10,13,15) were analyzed on 10% SDS-PAGE gels. Autoradiography was performed for 1 week.

Fig. 7. Histone H1 kinase activity of p55CDC immune complexes and phosphorylation of p55CDC.

(A) Lysates of CHO cell lines transfected with vector (PMT), vector with sense transcript (PMTp55s), and vector with antisense transcript (PMTp55as) were immunoprecipitated with affinity purified p55CDC antibody. Immune complexes were assayed for histone H1 kinase activity as described in Materials and Methods.

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(B) CHO cells were labelled with [^{32}P]-orthophosphate as detailed in Materials and Methods. Immune complexes obtained from 900 μg lysate precipitated with 1 μg of affinity purified p55CDC antibody (lane 1) or 28 μg of p55CDC competed antiserum (lane 2) were analyzed by SDS-PAGE.

Fig. 8. Immune complexes detected by p55CDC antibodies in Rat 1 and HeLa cells and their kinase activity against a variety of substrates at different stages of the cell cycle.

(A) Lysates (250 μg) from exponentially growing Rat 1 and HeLa cells were immunoprecipitated with p55CDC competed antiserum (lanes 1 and 3), affinity purified p55CDC antibody (lanes 2 and 4), and two different monoclonal antibodies against retinoblastoma protein (lanes 5 and 6).

(B) Lysates (200 μg) from HeLa cells prepared as described in Materials and Methods were immunoprecipitated with either control p55CDC competed antiserum shown in the first lane of each substrate or with affinity purified p55CDC antibody. Kinase assays were performed as described in Materials and Methods with decreasing exogenous substrate concentrations shown from left to right. The histone H1 concentrations in these assays was 0.4 mg/ml, 0.2 mg/ml and 0.1 mg/ml. Myelin basic protein (MBP) and α -casein concentrations decreased from 0.4 mg/ml to 0.1 mg/ml. The control assay was always performed using the highest substrate concentration.

(C) Lysates (200 μg) prepared from HeLa cells as described in Materials and Methods were immunoprecipitated with increasing amounts of affinity purified p55CDC antibody (0.07 μg , 0.28 μg and 1.12 μg). The negative control was done using 4.2 μg of the p55CDC competed antiserum. Kinase assays were performed as

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described in Materials and Methods using 0.4 mg/ml MBP as the substrate.

(D) Lysates (200 µg) were prepared from HeLa cells at various stages of the cell cycle as described in Materials and Methods and immuno-precipitated with either 8.4 µg p55CDC competed antiserum (lanes 1,8 and 9) or 1.0 µg of affinity purified p55CDC antibody (lanes 2-7). Kinase assays were performed using 0.4 mg/ml H1, 0.4 mg/ml MBP or 0.4 mg/ml α-casein as exogenous substrates.

(E) The excised bands from the dried gel obtained in Fig. 7D were counted. The control values (Fig. 8D lanes 1, 8 and 9) were subtracted from the experimental values (Fig. 8D lanes 2-7) and the results graphed.

Fig. 9. Cycling cells actively translate p55CDC and show high levels of associated α-casein kinase activity when compared to quiescent cells.

(A) Growing and quiescent Rat1 cells were labelled for one hour with 35S-Translabel as described in Materials and Methods. Lysates (100 µg) were immunoprecipitated with various antibodies. Immune complexes obtained with 10 µl of p34cdc2 MAb (lanes 1 and 7, p55CDC competed antiserum (8.4 µg/ lanes 2 and 8) and affinity purified p55CDC antibody (0.035 µg/ lane 3, 0.14 µg/lanes 4 and 9, 0.56 µg/ lanes 5 and 10, 1.12 µg/lanes 6 and 11) were analyzed by SDS-PAGE.

(B) Lysates (100 µg) were prepared from growing and quiescent Rat1 cells as described in Materials and Methods. Immune complexes were obtained with 10 µl p34cdc2 MAb, 8.4 µg of p55CDC competed antiserum and 1.12 µg of affinity purified p55CDC antibody. Kinase assays were performed as described in Materials and Methods using 0.4 mg/ml α-casein as substrate.

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DETAILED DESCRIPTION OF THE INVENTION

5 The present invention relates to a polypeptide designated p55CDC which is involved in mammalian cell division. mRNA transcripts encoding p55CDC were expressed in embryonic tissues and adult hematopoietic tissues which comprise populations of proliferating
10 cells, but were not detected in adult tissues lacking actively dividing cells. Moreover, human hematopoietic cell lines which were induced to differentiate with chemical agents also demonstrated loss of p55CDC transcript as cell division ceased. It has been
15 observed that polypeptides encoded by the rat p55CDC DNA sequence (Figure 2 and SEQ ID NO:1) and human p55CDC DNA sequence (Figure 2 and SEQ ID NO:3) have extensive amino acid sequence homology to portions of the cdc4 and cdc20 proteins from *Saccharomyces cerevisiae* (Figure 3). As
20 cdc4 and cdc20 are both known to be involved in mitosis and cell division, this homology has suggested involvement of p55CDC in these processes as well. Additional evidence implicating p55CDC in cell division is presented in Example 2. It was shown that down-
25 regulation of p55CDC expression by transfecting host cells with a rat p55CDC anti-sense clone resulted in surviving cells which overproduced sense transcripts, apparently to compensate for the loss of p55CDC mRNA. In addition, rat p55CDC appears to be synthesized at
30 high levels in actively growing cells, but not in quiescent cells (Example 5).

p55CDC appears to modulate mitosis and cell division through the formation of a complex with at
35 least one other host cell protein. Complexes containing p55CDC were precipitated by p55CDC antisera from Rat1

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cells, HeLa cells, and CHO cells transfected with a rat p55CDC clone. A presumed host cell protein was observed to be associated with p55CDC in each of these cell lines (Examples 3 and 4). The p55CDC complexes from these

5 cell lines displayed kinase activity which fluctuated during the cell cycle. The kinase activity of the p55CDC complex can be distinguished from the activities of other known cell cycle-associated kinases, including cyclin A/CDK2, cyclin E/CDK2 and cyclin B/p34cdc2

10 complexes, in the following ways: (1) p55CDC complexes had kinase activity against a number of substrates, including histone H1, myelin basic protein and α -casein rather than against a single substrate; and (2) a decrease in p55CDC-associated kinase activity was

15 observed at the G₁/S transition and at the G₂/M transition. This profile of cell cycle kinase activity has not been previously observed.

The invention provides for an isolated DNA

20 encoding a biologically active p55CDC polypeptide wherein the DNA is selected from the group consisting of:

- a) DNA having a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 2;
- 25 b) DNA having a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 4; and
- c) DNA having a nucleotide sequence which hybridizes with the DNA of (a) or (b), or with a fragment thereof, wherein the hybridizing DNA encodes a
- 30 polypeptide having the biological activity of p55CDC.

DNA of the present invention will preferentially hybridize to DNA sequences encoding p55CDC under appropriate conditions of temperature and salt. Establishment of appropriate hybridization

35 conditions is well within the ability of one skilled in the art using published protocols (see e.g., 63). As an

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example, hybridizations may be performed at 42° in 40% formamide and 5xSSPE for at least 12 hours, followed by three washes in 2XSSC, 0.1% SDS at 50° and one wash in 0.5xSSC, 0.1% SDS for 30 minutes. Sequences which
5 hybridize with p55CDC DNA will be related by deletion, insertion, point mutation, frameshift, alternative open reading frame, or mRNA splice variant. Hybridizing sequences may also be antisense nucleic acids (DNA or RNA) which bind to p55CDC DNA or RNA so as to modulate
10 the expression of p55CDC. Antisense nucleic acids may target the p55CDC coding region or regulatory sequences involved in transcription and/or translation of p55CDC.

DNA sequences hybridizing to p55CDC DNA will
15 preferably encode for a polypeptide having the biological activity of p55CDC. As shown in Examples 3 and 4, p55CDC associates with one or more host proteins to form a complex wherein said complex has cell cycle-dependent kinase activity. The biological activity of
20 p55CDC, as described herein, refers to a complex-associated kinase activity which is active on various substrates such as histone H1, α -casein and myelin basic protein, and wherein the kinase activity on one or more substrates is modulated during the cell cycle. For
25 example, the kinase activity of p55CDC complexes on α -casein is diminished during the G₁/S and G₂/M transitions of the mammalian cell cycle.

The invention also relates to a p55CDC
30 polypeptide as the product of procaryotic or eucaryotic expression of an exogenous DNA sequence, that is, p55CDC is preferably recombinant p55CDC. Exogenous DNA encoding p55CDC may be genomic DNA, cDNA, or may be partially or completely synthetic DNA. In one
35 embodiment, p55CDC DNA includes one or more codons which are preferred for expression in procaryotic host cells,

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especially *E. coli* host cells. Synthesis of DNA fragments for assembly into sequences for p55CDC expression is accomplished using synthetic methods which are readily available to one skilled in the art, such as
5 those described in Engels et al. (Angew. Chem. Intl. Ed. 28, 716-734 (1989)).

Also provided by the invention are plasmids and host cells for the expression of p55CDC protein.
10 p55CDC expression may be accomplished in procaryotic or eucaryotic hosts (e.g., mammalian, plant or insect cells, yeast or bacterial cells). Preferred host cells include mammalian cells, such as Chinese Hamster Ovary (CHO) cells, or bacterial hosts such as *Escherichia*
15 *coli*. p55CDC may be expressed from a variety of plasmid or viral vectors which are appropriate for the host cell being used. The use of vector pMT for the expression of rat p55CDC in CHO cells is described in Example 2. However, other vectors that are suitable for p55CDC
20 expression in other host cells may also be used. Expression of p55CDC in transgenic animals may be obtained using expression vectors and DNA transfection procedures available to one skilled in the art.

25 A method for producing a p55CDC polypeptide is also included. The method comprises culturing a procaryotic or eucaryotic host cell into which an expression vector containing a p55CDC DNA sequence has been transformed or transfected such that a p55CDC
30 polypeptide is expressed.

An isolated p55CDC polypeptide is encompassed by the present invention. Such polypeptides may be produced by expression of DNA molecules encoding p55CDC,
35 or they may be produced by chemical synthesis of peptides using procedures available to one skilled in

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the art. p55CDC polypeptides produced by the
aforementioned biological or chemical methods are
isolated using purification techniques which are known
to one skilled in the art. p55CDC polypeptides may be
5 analogs of rat or human polypeptides shown in SEQ ID NO:
2 or SEQ ID NO: 4, respectively, wherein said analogs
comprise the substitution, deletion or insertion of one
or more amino acids. In addition, chemical synthesis of
p55CDC polypeptides allows the inclusion of non-
10 naturally occurring amino acids (e.g., D-amino acids) at
selected positions. Amino acid residues within the
p55CDC polypeptide which are required for activity are
determined by generating analogs and testing said
analogues for activity, such as the ability to form a
15 complex having cell cycle associated kinase activity, or
the ability to advance a host cell through the cell
cycle. Protein kinase assays described in Materials and
Methods can be used to test for the biological activity
of p55CDC analogs. Selected regions of a p55CDC
20 polypeptide, such as those which show homology to the
cell division proteins cdc4 and cdc20 (see Figure 3),
may be used to design biologically active p55CDC analogs
or peptide fragments. These regions are referred to as
GB repeats and are likely to be important in the
25 structure and/or function of p55CDC.

Antibodies specifically binding p55CDC
polypeptides of the invention are also provided.
Antibodies may be polyclonal or monoclonal and may
30 recognize fragments, analogs and fusion polypeptides of
p55CDC as well as the intact protein. Mouse anti-p55CDC
antibodies may be produced by techniques available to
one skilled in the art and may be modified to form
chimeric or humanized antibodies. Anti-p55CDC
35 antibodies are useful in assays described below for

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quantitating p55CDC and p55CDC complexes that are present in biological samples.

A complex comprising p55CDC and at least one other host cell protein is also provided. Example 3 describes an immune complex from tranfected CHO cells having p55CDC and an associated 210 kDa protein wherein the complex has cell cycle associated kinase activity. Example 4 describes immune complexes from rat 1 and Hela cells which have p55CDC associated with a second polypeptide and exhibit kinase activity. A 110 kDa protein was identified in rat immune complexes and a 100 kDa protein was identified in Hela immune complexes. The ability of p55CDC to associate with at least one other polypeptide such that the resulting complex phosphorylates various host cell molecules appears to correlate with the ability of p55CDC to modulate the cell cycle. Also encompassed by the invention are complexes comprising a p55CDC analog and at least one other host cell protein. In a preferred embodiment, p55CDC complexes will have cell cycle dependent kinase activity such as that described in Example 4.

The invention also relates to a method for detecting levels of p55CDC in biological samples. The method comprises incubating an antibody specifically binding p55CDC, or a fragment, analog, or fusion polypeptide thereof, with a sample under conditions suitable for forming a complex between the antibody and p55CDC and detecting the presence of a p55CDC-antibody complex. The antibody may also bind to p55CDC when p55CDC is complexed with other host cell proteins. Therefore, the method also encompasses the detection of p55CDC complexes. Since p55CDC is present in actively dividing cells, but not in quiescent cells, it is anticipated that a diagnostic assay for p55CDC will be

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most useful in identifying those samples having elevated levels of cell division.

Methods for modulating cell division are also provided. It will be appreciated by one skilled in the art that compounds which modulate p55CDC activity will modulate cell cycle activity as well. Compounds which modulate the synthesis of p55CDC and/or modulate the ability of p55CDC to form a complex having cell cycle associated kinase activity may be identified using the procedures described for determining p55CDC activity. Modulation of p55CDC kinase activity may involve an increase or decrease in activity at certain periods during the cell cycle which may lead to alterations in the timing or specificity of p55CDC complex activity, Compounds which in turn may be used to control cell division include, but are not limited to, the following: (1) compounds which increase or decrease the levels of p55CDC synthesis; (2) compounds which bind to p55CDC so as to interfere with formation of a p55CDC complex having kinase activity; (3) compounds which compete with p55CDC for complex formation and themselves form inactive complexes; and (4) compounds which promote the formation of the p55CDC complex or stabilize said complex from dissociation by increasing the half-life. Examples include nucleic acid molecules which bind to p55CDC DNA or p55CDC polypeptides, antibodies, peptides, organic molecules, and carbohydrates. Such compounds are identified by screening large repertoires, or libraries, comprising nucleic acids, peptides or small organic molecules derived from chemical synthesis or natural sources (e.g., bacteria, fungi, plants). Considerable literature exists on the synthesis, characterization and screening of very large natural or synthetic libraries of molecules or polymers. One skilled in the art would appreciate that such libraries

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can be screened for compounds that modulate p55CDC activity.

Compounds which inhibit the biosynthesis or
5 activity of p55CDC are useful in inhibiting the growth
of those tumor cells having increased levels of p55CDC
or increased levels of cell cycle dependent kinase
activity associated with p55CDC as compared to normal,
noncancerous cells. Compounds useful as
10 chemotherapeutic agents include, but are not limited to,
the following: (1) compounds which decrease the levels
of p55CDC synthesis; (2) compounds which bind to p55CDC
so as to interfere with formation of a p55CDC-host cell
protein complex having kinase activity; and (3)
15 compounds which compete with p55CDC for association with
one or more host cell proteins involved in complex
formation and themselves form inactive complexes. Tumor
cells which grow more rapidly than normal, noncancerous
cells perhaps by virtue of increased p55CDC activity may
20 be more responsive to p55CDC-inhibiting agents. Such
agents would be expected to have less effect on p55CDC
activity in normal cells.

A method of chemotherapy comprising treating a
25 mammal with an amount of a compound which is effective
in reducing or inhibiting p55CDC activity in a
pharmaceutically effective adjuvant is also provided.
Compounds which reduce or inhibit p55CDC activity are
identified by screening appropriate sources for activity
30 against p55CDC using assays for p55CDC activity as
described herein. A dosage which is effective in
reducing or inhibiting p55CDC activity may be determined
by one skilled in the art taking into account such
factors as the condition being treated and
35 administration regimen. Important considerations
include the type and location of the tumor being treated

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and whether the route of administration is by injection (intravenous, intramuscular, or subcutaneous) or by oral or nasal intake. Compounds of the present invention are mixed with a pharmaceutically acceptable adjuvant which
5 may include any suitable buffer, solubilizer, preservative, carrier or anti-oxidant. Preferably, the adjuvant will not decrease the p55CDC-inhibiting activity of the compound. An extensive survey of pharmaceutically acceptable ajuvants is found in
10 Remington's *Pharmaceutical Sciences*, 18th ed. A.R. Gennaro, ed. Mack, Easton, PA (1990).

EXAMPLE 1

15 IDENTIFICATION AND CHARACTERIZATION OF p55CDC

The p55CDC gene

The gene encoding p55CDC was identified serendipitously during an attempt to identify novel
20 glycosyltransferase enzymes by low stringency screening of a rat genomic library with cDNA encoding the rat $\alpha 2,6$ sialyltransferase (57,73). During one round of screening, a genomic clone was isolated. Restriction map analysis first narrowed the hybridizing region to a
25 2 kb *Bgl* II fragment. The cross hybridizing region of this fragment was further narrowed to a 0.26 kb *Pst* I fragment which was used for Northern analysis of various embryonic, neonatal and adult rat tissues.. This
identified a tissue which could be used as a source of
30 RNA for construction of a cDNA library.

Northern analysis revealed tissue-specific and developmentally-regulated expression of a unique transcript (Fig. 1A). A 2 kb mRNA was abundant in RNA
35 from total rat embryo, and this transcript was enriched in embryonic rat liver. However, in two day old

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neonatal rats, the level of message in the liver decreased precipitously. The transcript was still abundant in spleen from 2 day old rats, and a small amount was present in kidney. In 16 day old rats, the transcript was still abundant in spleen and thymus, but was barely detectable in liver and kidney. The transcript was not detectable in any adult tissues, although a longer exposure of a blot containing more RNA did reveal a faint band in the spleen sample. The presence of the transcript in hematopoietic tissues, such as neonatal liver, thymus and spleen, suggested that expression of this novel gene was highest in tissues in which cell proliferation was occurring.

A cDNA library was constructed using polyA+ RNA from two day old rat spleen. Using the *Pst* I genomic fragment as a probe, several positive plaques were identified, at a frequency of approximately 1:15,000. The two largest cDNA inserts were subcloned and sequenced. The nucleotide sequence (Fig. 2 and SEQ ID NO: 1) coded for a protein of 499 amino acids, with a predicted molecular mass of 55 kDa. However, this sequence did not appear to encode a classical glycosyltransferase enzyme, since there was no evidence of an amino-terminal hydrophobic membrane spanning signal-anchor domain, which is essential for glycosyltransferases to be properly oriented in the Golgi (57).

The human p55CDC gene was isolated from an HT1080 cell line cDNA library by the following procedures. The nucleotide sequence and deduced amino acid sequence are shown in Fig. 2 and in SEQ ID NO: 3. A comparison of the open reading frames of the rat and human sequences showed an 87% identity at the nucleotide level, which increased to 95% at the amino acid level.

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Differences in the human nucleotide sequence are shown above the rat sequence, and differences in the human amino acid sequence are shown below. The human sequence diverged considerably from the rat upstream of the ATG start site, and also in the 3' untranslated region.

Homology of p55CDC to cell cycle proteins

A search of the genEMBL database revealed that rat and human p55CDC proteins had seven regions of homology with the WD-40 repeat of the β subunit of G proteins (27) (Fig. 3A), and to a number of proteins which contain this imperfect repeat motif (for review, see 12,72). These included the products of the *S. cerevisiae* genes *CDC20* (65) and *CDC4* (77), *TUP1/AER2* (78), *PRP4* (58), and *MSI1* (62), as well as the products of the *D. melanogaster* gene *Espl*, the *D. discoïdum* gene *AAC3* (66), the *Arabidopsis thaliana* gene *COP1* (13) and the dTAF₁₁₈₀ subunit of *Drosophila* TF11D (22). The highest degree of homology, illustrated in Fig. 3B, was seen between p55CDC and the two *S. cerevisiae* cell division cycle proteins, Cdc20 (519 amino acids) and Cdc4 (779 amino acids). The BESTFIT analysis revealed a 45% identity between amino acids 172-407 of p55CDC and amino acids 249-479 of the Cdc20 protein, which increased to 59% when highly conserved substitutions were included. This was the only protein in which a high degree of similarity was found with the degenerate internal GB-repeats in p55CDC. The Cdc4 protein was the only protein which showed strong homology with all seven repeats found in p55CDC, using the first seven of the nine repeats found in the Cdc4 protein (Fig. 3B). The alignment of the highly degenerate WD-40 repeats in these two proteins required the introduction of 16 gaps over 300 amino acid residues. This comparison indicated that 28% of the residues in this region were identical, and 41% were identical or highly conserved. Notably,

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the *S. cerevisiae* Cdc20 and Cdc4 proteins each displayed a greater degree of homology to the mammalian p55CDC protein than they did to each other.

5 Recently a clone isolated from a *Xenopus*
oocyte cDNA library by virtue of its ability to suppress
the temperature sensitive defect of the *S. Cerevisiae*
cdc15 mutation, was shown to encode a protein of 518
amino acids that has seven G β -repeats in its carboxy
terminal half (69). This protein called β TrCP (β -
10 transducin repeat containing protein) was not a
functional homolog of *CDC20*, though overexpression of
both these genes is capable of suppressing the *cdc15*
mutation (1,69). Both β TrCP and p55CDC have seven G β -
repeats and show 24% identity over this region.

15 The only protein that showed significant
homology to p55CDC extending beyond the G β - repeats was
the *S. cerevisiae* MSI1 protein, which is a negative
regulator of the RAS-mediated induction of cAMP levels
(62). The MSI1 protein (422 amino acids) was 24%
20 identical to p55CDC, and this increased to 28% when only
the amino terminal 178 residues of p55CDC were compared
to the amino terminal 148 residues of MSI1.

Cross species homology of p55CDC

25 A high degree of evolutionary conservation
within the p55CDC open reading frame was seen when
genomic DNA from a variety of mammalian species,
chicken, *D. melanogaster* and *S. cerevisiae* were examined
by Southern blot analysis, using the rat cDNA probe
30 (Fig. 4). A cross-hybridizing species was detectable in
all the mammalian and the avian species, although no
bands were seen in the lanes containing *S. cerevisiae*
and *D. melanogaster* DNA. These results also indicate
that the gene encoding p55CDC is a single copy gene,
35 with no closely related genes in the species examined.

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Expression of p55CDC in tissues and cell lines

The pattern of expression of p55CDC mRNA in embryonic and neonatal rat tissues, and the apparent relationship between p55CDC and the *S. cerevisiae* Cdc20 and Cdc4 proteins suggesting a possible role for p55CDC in cell division, prompted us to examine other developing mammalian tissues for p55CDC expression. Northern analysis of human tissues demonstrated a pattern of expression similar to that seen in the rat, with high levels of expression in fetal liver and juvenile thymus, but no expression seen in fetal lung, adult lung or liver, or adult buffy coat, which is primarily comprised of non-dividing white blood cells (Fig. 1B). A second Northern analysis examining polyA⁺ RNA from adult human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas demonstrated expression of p55CDC in only one tissue, the placenta, which contains actively dividing cells; a similar pattern of expression has been described for p34cdc2 (48).

A number of human cell lines also expressed the p55CDC transcript. The transcript was abundant in all leukemia cell lines examined, including the T cell lines MOLT 4f and CEM, the B cell lines Raji and Ramos, the monocytic cell line U937 and the myeloerythroid cell line K562 (Fig. 1C). Indeed, we observed expression of the p55CDC transcript in every cell line examined at log phase of growth, regardless of lineage.

To examine whether expression of the p55CDC transcript was related to the ability of cells to divide, we took advantage of the unique properties of two leukemia cell lines, K-562 and HL-60. K-562 cells can be induced by treatment with sodium butyrate to undergo erythroid differentiation with no significant

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effect on growth rate (2). In contrast, treatment of K-562 cells with the phorbol ester TPA causes monocytic differentiation accompanied by growth arrest (5). Treatment of HL-60 cells with TPA also causes monocytic differentiation, with arrest of DNA synthesis and cell division (61). We examined the effects of these agents on the level of expression of p55CDC mRNA in these two cell lines (Fig. 1D). The p55 transcript was easily detectable in both mock-treated cell lines. For both K-562 and HL-60, treatment of the cells with TPA resulted in loss of p55CDC mRNA expression. In the K-562 cells treated with sodium butyrate, in which differentiation is not accompanied by growth arrest, the level of p55CDC transcript was roughly equal to that found in the mock-treated cells. These results indicate that p55CDC mRNA is synthesized only in dividing cells.

EXAMPLE 2

EFFECTS OF p55CDC ON CELL PROLIFERATION

To explore possible functions of the p55CDC protein, CHOd- cells were transfected with plasmid containing the cDNA encoding the rat p55CDC in either the sense (PMTp55s) or antisense (PMTp55as) orientation. A 1.8 kb fragment of rat cDNA was inserted downstream of the metallothionein promoter in the pMT010/A+ mammalian expression vector (9). This vector also contains two dominant selectable markers, the bacterial *neo* gene and the mouse *DHFR* gene, driven by the SV40 promoter. Control cells were transfected with vector alone (PMT). Following amplification with methotrexate, the three pools of cells were plated at a density of 0.5×10^6 cells per 60 mm plate in the presence of 0.05 mM zinc, and the growth profiles plotted for fourteen days (Fig. 5A). Pools of transfected cells were studied, rather than

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individual clones, to minimize the effect of any clonal variation in the CHOD⁻ cells. Initially, little difference in the growth rates were observed among the three pools of transfected cells, although the PMTp55as
5 cells were significantly larger than the PMTp55s or control cells as shown by forward scatter analysis determined by flow cytometry and visual observation under the microscope. Moreover, a DNA content analysis as measured by propidium iodide uptake showed that
10 PMTp55as cells had an increased amount of DNA/cell, indicating that these cells were hyperdiploid (Fig. 5B). As the plates began to reach confluence, dramatic differences in the growth profiles were observed. The PMTp55 as cells reached confluence first, at a lower
15 cell number, consistent with their larger size. After reaching confluence the PMTp55as cells continued to divide slowly. The smaller PMTp55s cells continued to divide at a faster rate after reaching confluence. The PMTp55s cells reached a density of 24×10^6 cells/plate by
20 day 14, compared to 6×10^6 cells/plate for the PMTp55as cells. The growth profile of the PMT cells fell midway between those of the PMTp55s and PMTp55as cells.

Since the cells transfected with the vector
25 encoding an antisense transcript continued to survive, although with an altered phenotype, we examined the pools of transfected cells for the presence of sense and antisense p55CDC mRNA transcripts using an RNase protection assay (70). As shown in Table 1, the PMT
30 cells had an average of 166 copies of sense mRNA per cell, while, as expected, the PMTp55s cells had an increased average of 734 copies of sense mRNA per cell. Surprisingly, the PMTp55as cells also had an increased number of copies of sense mRNA, with an average of 714
35 copies per cell. In addition, the PMTp55as cells had only a moderate amount of antisense mRNA, with an

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average of 205 copies per cell, despite having been transfected with cDNA encoding the antisense transcript. This same pattern was observed when clonal cell lines isolated from the pools of cells were analyzed. Each of the four PMTp55as clonal lines made elevated amounts of the sense transcript; in all lines, this amount was at least five times the amount of the antisense transcript. As expected, in the control PMT cells, the average number of copies of sense transcript per cell declined considerably in confluent cells.

TABLE 1
p55CDC mRNA copy number in both sense and antisense orientation in various cell lines

Cell Line	48 hours mRNA copies/cell ^a		7 days mRNA copies/cell ^a	
	Sense	Anti-sense	Sense	Anti-sense
PMT	166 ± 6	-	4 ± 7	-
PMTp55s	734 ± 40	21 ± 7	240 ± 9	16 ± 5
PMTp55as	714 ± 10	205 ± 10	263 ± 10	96 ± 10
PMTp55A2s	771 ± 12	2	ND ^b	ND
PMTp55B6s	4136 ± 66	117 ± 4	ND	ND
PMTp55B12as	706 ± 20	126 ± 3	ND	ND
PMTp55G6as	1176 ± 10	213 ± 14	ND	ND
PMTp55H5as	928 ± 17	157 ± 2	ND	ND
PMTp55H11as	1149 ± 21	128 ± 7	ND	ND

a. Values were determined as described in experimental procedures.

b. Not Determined

Genomic DNA analysis of all six clonal cell lines demonstrated that the elevated expression of sense

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transcripts was not due to amplification of the endogenous gene. The two clonal isolates expressing sense orientation transcripts were distinct from one another. In contrast, it is likely that all four of the

5 PMTp55as clones we isolated derived from the expansion of only one transfected cell in the original pool of cells, since restriction map analyses of the four clonal PMTp55as cell lines using two different restriction

10 enzymes and two different probes, to detect either plasmid or p55CDC sequences, demonstrated identical banding patterns. The results indicated that inhibition of p55CDC expression by antisense transcripts was compensated for by overexpression of sense transcripts. This data suggested that p55CDC was essential for

15 maintenance of cell proliferation in culture.

EXAMPLE 3

IMMUNE COMPLEXES CONTAINING p55CDC

20 Polyclonal rabbit antiserum was raised against a fusion protein consisting of p55CDC and glutathione S-transferase. Both the original antiserum and an affinity purified antibody preparation precipitated a

25 protein of M_r 55 kDa from an *in vitro* transcription/translation reaction containing p55CDC cDNA, consistent with the predicted mass of the polypeptide.

30 To examine the level of p55CDC production in the transfected cell lines, immunoprecipitations were performed on extracts of ^{35}S -labelled cells in log phase, using the affinity purified antibody. As shown in Fig. 6A, the PMTp55s and PMTp55as cells had increased

35 levels of p55CDC compared to the PMT cells, consistent with the demonstration of increased numbers of

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transcripts encoding p55CDC in these cells. In the PMTp55s cells, there was a strong band of 31 kDa which probably represents a degradation product of p55CDC, since this band was also detected on immunoblot analyses of cell extracts using the polyclonal antibody preparation. This 31 kDa band was also observed when cell lysates were prepared without protease inhibitors, and no intact p55CDC was detected in the absence of protease inhibitors. This peptide was not p34cdc2, since no p34cdc2 protein was detected in any of the immune complexes.

Immunoprecipitates of p55CDC also contained a protein of M_r 210 kDa. The amount of p210 detected in the immunoprecipitates was roughly proportional to the amount of p55CDC. When this experiment was repeated on cells in stationary phase, seven days after plating, a significant decrease in the amounts of both p55CDC and p210 is observed (Fig. 6B); in Fig. 6B, a one week exposure of the autoradiogram was required to detect p55CDC, compared to a 21 hour exposure in Fig. 6A. These results indicate that production of p55CDC is highest in proliferating cells.

EXAMPLE 4

KINASE ACTIVITY OF p55CDC IMMUNE COMPLEXES

Since many events in the cell cycle are controlled by various kinases, it was of interest to determine whether p55CDC immune complexes had any kinase activity. All immune complexes examined for protein kinase activity were precipitated under conditions identical to those used in Fig. 6. The immunoprecipitation buffer was formulated (1% NP-40, 1% deoxycholate and 0.1% SDS) to minimize non-specific

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protein association. A number of cell division kinases can phosphorylate histone H1, so this substrate was assayed first. As shown in Fig. 7A, immune complexes precipitated with p55CDC antibody phosphorylated histone H1. Immune complexes prepared from lysates of the PMT, PMTp55s and PMTp55as cells all demonstrated kinase activity against histone H1. The highest levels of phosphorylation were seen in the PMTp55s and PMTp55as cells which have increased expression of p55CDC. In the negative controls, using competed antiserum, a small amount of residual activity is seen. In reactions performed without addition of exogenous substrates, no phosphorylated proteins were detected, indicating that none of the proteins in the immune complex are endogenous substrates of the kinase activity. However, when all three pools of transfected cells were labelled with [³²P]- orthophosphate and p55CDC was immunoprecipitated, SDS-PAGE analysis revealed that p55CDC was phosphorylated (Fig. 7B). Thus, p55CDC is a substrate of another endogenous kinase in the CHO cells. In the PMTp55s cells, no ³²P-labelled 31 kDa band was detected (see Fig. 6A and B, lanes 8 and 10), indicating that the 31 kDa degradation fragment of p55CDC is either not phosphorylated, or is dephosphorylated prior to degradation.

We wished to examine whether p55CDC in different cell lines was associated with other proteins in immune complexes and whether these complexes also had kinase activity. The Rat1 fibroblast and HeLa cell lines were chosen for this analysis. Proliferating Rat1 and HeLa cells were lysed, and immune complexes precipitated with the affinity purified p55CDC antibody (Fig. 8A, lanes 2 and 4). SDS-PAGE analysis of the immune complexes did not reveal the 210 kDa band seen in CHO cells, but did reveal other discrete bands which

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appeared to be cell-specific. In the Rat1 cells, a protein of 110 kDa was present in the p55CDC immune complexes, while a protein of 100 kDa was seen in p55CDC immune complexes in HeLa cells.

5

The p55CDC immune complexes from HeLa cells were examined for kinase activity against a number of different substrates (Fig. 8B). Kinase activity was detected with histone H1, myelin basic protein and α -casein, with maximal activity detected with myelin basic protein. β -casein was also examined, but minimal activity was detected with β -casein as a substrate (data not shown). The level of kinase activity correlated with p55CDC concentration, since increasing the amount of antibody used for the immunoprecipitation resulted in increased phosphorylation of myelin basic protein (Fig. 8C).

To determine whether the p55CDC-associated kinase activity fluctuated during the cell cycle, as has been described for the cyclin-dependent kinases, cells were arrested at various points in the cell cycle and immune complexes precipitated from cell lysates were examined for kinase activity. A distinct pattern of a cell cycle related fluctuation in kinase activity was detected with only one of the three substrates examined, α -casein (Figs. 8D and E). Kinase activity against α -casein was present in HeLa cells and in cells blocked in G₁ by serum starvation. The level of activity against α -casein dropped approximately four-fold in cells arrested at G₁/S and returned to the higher levels in cells harvested during S phase. Kinase activity remained constant in cells in the G₂ stage of the cell cycle and decreased six-fold in cells at the G₂/M transition. Kinase activity against histone H1 by p55CDC immune complexes was stable throughout the cell

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cycle (Fig. 8E). Background levels of histone H1 kinase activity in the G₂/M cells (Fig. 8D, lane 8) was most likely due to residual p34^{Cdc2} kinase activity in these samples. Kinase activity against myelin basic protein was also relatively constant throughout the cell cycle, with the exception of the G₂/M transition, where a two-fold decrease in activity was observed. While p55CDC was difficult to detect by immunoblotting of cell lysates prepared from cells at any of the various stages, the amount of p55CDC present in cells did not appear to fluctuate during the cell cycle, in contrast to the fluctuation in kinase activity observed with p55CDC immune complexes.

15

EXAMPLE 5

p55CDC EXPRESSION AND KINASE ACTIVITY IN GROWING AND QUIESCENT CELLS

The expression of p55CDC and the associated kinase activity in growing and quiescent populations of cells was compared by exploiting the ability of Rat1 cells to arrest growth under limiting serum conditions. As shown in Fig. 9A, exponentially growing Rat1 cells actively synthesized labelled p55CDC (lanes 3-6), while the quiescent population showed minimal production of p55CDC within the one hour labelling period (lanes 9-11). To rule out that the kinase activity we observed was precipitated non-specifically from the cell lysates, increasing amounts of p55CDC antibody were used in the immunoprecipitations. As shown in lanes 3-6, increasing the amount of p55CDC antibody resulted in the precipitation of increasing levels of p55CDC. This result is consistent with the result observed in Fig. 8C, where increasing the amount of antibody used for precipitation increased the level of p55CDC kinase

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activity detected. The production of labelled p34cdc2 is also substantially reduced in the quiescent population (compare lanes 1 and 7), although the amount of total p34cdc2 in the two samples was virtually equivalent, as detected on Coomassie blue stained gels of immunoprecipitated material. We also examined the p55CDC associated kinase activity under these two conditions and compared it to that observed for p34cdc2 immune complexes as a control. A higher level of activity was observed with the p55CDC complexes using α -casein as a substrate, since α -casein is a poor substrate for the p34cdc2 kinase (Fig. 9B). Both the p34cdc2 kinase and the p55CDC associated kinase showed a decrease in activity in the quiescent cells. As seen in the HeLa cells, when myelin basic protein was used as a substrate, no significant change in the p55CDC associated kinase activity was observed.

20

MATERIALS AND METHODS

RNA Analysis

Total RNA was prepared from freshly dissected rat tissues, human thymus and buffy coat, by the method of Chomczynski and Sacchi (8). mRNA from human cell lines was prepared by the Fastrack kit (Invitrogen). Gel electrophoresis of total RNA (30 μ g/lane) was done in 1% agarose gels containing formaldehyde and Northern hybridizations were performed as reported earlier (73). Radiolabelled probes were generated using the Amersham Multiprime DNA labelling system RPN.1601. mRNA size was determined by comparing with commercial RNA standards (Bethesda Research Laboratories, Gaithersburg, MD). mRNA from other human tissues was purchased from Clontech as was a multiple human tissue Northern blot.

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To generate the riboprobes for the RNase protection assay, the gel purified p55CDC cDNA fragment was subcloned into Bluescript (Stratagene, LaJolla, CA) in both the sense and antisense orientation relative to the T7 promoter. All subsequent steps were performed as described previously (70). Briefly, cells (1×10^6 /ml) were washed in phosphate buffered saline (PBS) and lysed by incubation at room temperature for 20 minutes in 10mM Tris pH8.0, 1mM EDTA, 20mM dithiothreitol, 100 μ g/ml proteinase K and 0.2% SDS. Lysed samples were added to hybridization mix with the labelled riboprobe and incubated at 84° for 2 hours. Following RNase digestion for 20 minutes at 37° using RNase A and RNase T1, the sample was loaded onto a Sephacryl S200 Superfine gel filtration column (Sigma, St. Louis, MO) and the void volume fraction containing the protected probe was counted. The quantity of gene specific RNA was calculated from a standard curve. All assays were performed in duplicate.

20

DNA Analysis

Genomic Southern and restriction map analysis were performed using standard molecular biology techniques (63). Genomic DNA from various species was purchased from Clontech (Palo Alto, CA). Medium stringency hybridizations were performed at 42° in 40% formamide. All hybridizations were performed at a salt concentration of 5xSSPE. Following overnight hybridizations the filters were washed three times in 2XSSC, 0.1% SDS at 50°. The final wash was done in 0.5XSSC, 0.1% SDS for 30 minutes. DNA sequence was determined using Sequenase (U.S. Biochemical), following the manufacturer's protocol. Sequencing was also performed on the Applied Biosystems 373A automated DNA sequencer using the Tag Dye Deoxy Terminator kit according to suggested protocol. The comparative

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percent identity values between the genes carrying the GB motif were obtained using the GCG BESTFIT program with gap weight set at 2.0 and length weight at 0.05.

5 CDNA Cloning of Rat p55CDC

A rat genomic library made from a partial EcoRI digest ligated into Charon 4A (Clontech) was screened at low stringency (hybridizations performed in 43% formamide at 37°) with a 435 base pair cDNA probe
10 encompassing amino acid residues 141-286 of the $\alpha_2,6$ sialyltransferase gene (73). Restriction map analysis of the isolated genomic clone revealed a 2 kb Bgl II fragment that hybridized to the probe. This fragment was subcloned into a pUC vector and further analysis
15 narrowed the hybridizing region to a 0.26 kb Pst I fragment which was used in all subsequent analysis. PolyA⁺ RNA from newborn rat spleen was selected by two cycles of binding to oligo (dT)-cellulose type 2 (Collaborative Research). A cDNA library was
20 constructed using the Pharmacia cDNA synthesis kit followed by ligation into the lgt10 vector. This was packaged using the Gigapack II Gold cloning kit (Stratagene). An initial packaging reaction gave 3.3×10^6 pfu and 1×10^6 pfu were screened using the 0.26 kb
25 Pst I fragment as probe.

CDNA Cloning of Human p55CDC

The human HT1080 cell line cDNA library was constructed in the pSPORT-1 plasmid vector (BRL Life
30 Technologies, Inc.). DNA from 44 pools of approximately 5000 colonies each was linearized with Not I and screened by Southern blot, using the rat p55CDC cDNA as a probe. Plaque and colony purification of the clones with the longest inserts was done using standard
35 techniques (63).

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Cell Culture, Synchronization and Labelling

HL60 and K562 cells were grown in RPMI1640 (Irvine Scientific, Irvine, CA) supplemented with 10 mM HEPES and 15% fetal calf serum. Cells were seeded at a concentration of 0.2×10^6 cells/ml media. Cells treated with 1mM sodium butyrate were grown in 75 cm² flasks for three days. Cells induced with phorbol ester were grown in the presence of 30 ng/ml 12-O-teradecanoyl-phorbol 13-acetate (TPA) for three days. Cells were lysed by guanidine thiocyanate and total RNA prepared as described (8). CHOd- cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, glutamine, non-essential amino acids and hypoxanthine. Rat 1 cells were maintained in DMEM containing 10% serum and glutamine and HeLa cells in a Minimum Essential Medium supplemented with 10% serum, glutamine and non-essential amino acids.

HeLa cells were synchronized at the beginning of S phase (G₁/S) by the double thymidine/aphidicolin block described by Heintz et al (35). Cells harvested 4 hours later were in S phase (59). Synchronization at the G₂/M transition was achieved by growth in the presence of 0.5 µg/ml nocodazole for a period of 12-14 hours. The media was carefully suctioned off and the non-adherent mitotic cells were harvested by gently pipetting buffer onto the monolayer. The adherent cells were washed with PBS and then lysed. This population is not mitotic and is predominantly in G₂ (36).

Asynchronous exponentially growing cells were grown in methionine and cysteine free media containing 2% dialyzed serum for one hour followed by two hours in the same media containing 100 µCi/ml media of ³⁵S Translabel (ICN Biomedicals, Irvine, CA). [³²P]-

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orthophosphate (ICN Biomedicals) labelling was performed for 3 hours following a one hour preincubation in phosphate deficient media.

5 Rat1 cells were growth arrested by rinsing the plates with PBS, followed by rinsing in media containing 0.1% fetal calf serum. The cells were grown in the low serum media for 48 hours to obtain a quiescent population. Labelling with ³⁵S-Translabel was performed
10 as described above, with the exception that the dialyzed serum concentration was maintained at 0.1% and the label was incorporated over one hour. For the exponentially growing population of Rat1 cells, the dialyzed serum concentration was maintained at 2% during the course of
15 the labelling.

For flow cytometry analysis, 1×10^6 cells were washed in PBS and fixed in 70% ethanol, 2.0% Triton X-100 for one hour. Fixed cells were washed in PBS and
20 stained in a solution of 50 µg/ml propidium iodide (PI) and 20 µg/ml RNase A. The cells were analysed for DNA content (fluorescent intensity) and cell size (forward scatter) using the FACScan (Becton Dickinson, Mountain View, CA).

25

Transfection of CHOd- cells.

A 1.8 kb cDNA obtained from the newborn rat spleen library was cloned into the *Bam* H1 site of the pMT010/A+ mammalian expression vector (9). The cDNA was
30 inserted downstream of the metallothionein promoter in both the sense (PMTp55s) and antisense (PMTp55as) orientations. These plasmids, as well as vector alone as control, were transfected into cells using Lipofectin (BRL Life Technologies) following the suggested
35 protocol. Initial selection by Geneticin at 400 µg/ml in media without hypoxanthine was followed by stepwise

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amplification by methotrexate to a final concentration of 2 μ M. Growth curves were performed in media containing 0.05mM zinc to induce the metallothionein promoter.

5

Antibody Preparation

A p55CDC cDNA clone lacking the first 10 codons was inserted into the *Eco*R1 site of the pGEX-3X vector (Pharmacia GST gene fusion system). Competent
10 XL-1 cells (Stratagene) were transformed and a colony harboring the recombinant plasmid was isolated. Cultures were induced with isopropyl β -D-thiogalactopyranoside (final concentration 0.1 mM) for growth of the fusion protein. A 76 kDa insoluble fusion
15 protein was obtained which stayed with the pellet following sonication and solubilization with 1% Triton CF-54. The pellet obtained from lysed and sonicated cells was washed twice with PBS containing 1% Triton CF-54 and the resulting pellet extracted with 10M urea.
20 All attempts to extract the fusion protein in anything short of 10M urea failed. The urea extract was dialyzed overnight against PBS and the resulting suspension stored in aliquots at -80°C until further purification by SDS-PAGE electrophoresis. The pellets were
25 resuspended in SDS sample buffer and separated in 10% SDS gels. The region between the visible standard markers of 55 kDa and 80 kDa was excised out and the protein recovered by electroelution (Biorad Model 422 ElectroEluter). This preparation was mixed with
30 Freund's complete adjuvant and used for immunization of rabbits. A booster injection was given after four weeks using Freund's incomplete adjuvant. Animals were bled 10-14 days following a booster injection. To obtain an affinity column for purification of antiserum, the crude
35 insoluble fusion protein pellet was resuspended in coupling buffer (0.1M NaHCO_3 pH8.3, 0.5M NaCl, 0.5% SDS)

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and coupled to cyanogen bromide-activated Sepharose, according to the manufacturer's instructions (Pharmacia, Piscataway, NJ). A coupling efficiency of approximately 0.4 mg protein/ml gel was achieved. The antiserum was first absorbed against an unrelated insoluble fusion protein to remove any antibodies reactive against glutathione S-transferase or contaminating *E. coli* proteins. This partially purified antiserum was applied to the affinity column. The column was washed with 5x column volumes of PBS and the affinity purified antibodies were eluted with 3M sodium thiocyanate. Pooled antibody fractions were immediately dialyzed against PBS and stored at -80°C. The flowthrough fraction from this column was used as competed antiserum.

Immunoprecipitations And Protein Kinase Assays

In vitro translation was performed using a nuclease treated rabbit reticulocyte lysate (Promega, Madison, WI) and [³H]-leucine (Amersham TRK683). The mRNA template was produced by using the Stratagene in vitro transcription kit and the p55CDC cDNA subcloned into the Bluescript vector as substrate. Cell lysates were prepared as described (59) after rinsing the plates twice with PBS. Cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer with additional proteases (150 mM NaCl, 1.0% NP-40, 1.0% sodium deoxycholate, 0.1% SDS, 2mM EDTA, 6mM Na₂HPO₄, 4mM NaH₂PO₄ 50mM NaF, 200 μM Na₃VO₄, 20 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor and 50 μg/ml phenylmethyl sulfonyl fluoride). All protease inhibitors were purchased from Sigma. Protein concentrations were estimated using the Bicinchoninic acid reagent (Pierce). For 250 μg lysate in a final volume of 700 μl RIPA buffer, we used 7 μl of affinity purified p55CDC antibodies (140 μg protein/ml) or 12 μl

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of p55CDC competed antiserum (700 µg protein/ml) which gave an equivalent level of immunoglobulin for both preparations. Immunoprecipitation of p34cdc2 complexes was done using 10 µl of the p34cdc2 mouse monoclonal antibody 17 (Santa Cruz Biotechnology, Santa Cruz, CA). Other antibodies used in this study were Rb(1F8), a mouse monoclonal IgG against a Rb-β galactoside fusion protein (Santa Cruz Biotechnology) and Rb(Ab-1), another monoclonal antibody against retinoblastoma protein (Oncogene Science, Uniondale, NY). The immune complexes were routinely incubated overnight on ice and collected next morning with 30 µl of a 50% slurry of Protein G-Sepharose (Pharmacia). The washed pellets were assayed for histone H1 kinase activity as described in (59). All reactions were performed for 30 minutes at 30°. Assays were also performed using a variety of kinase substrates at the indicated concentrations, using the same assay conditions. Histone H1 was purchased from Boehringer Mannheim while myelin basic protein (MBP), β-casein and α-casein were all purchased from Sigma. The reaction products were quantitated by excising the stained bands from the dried gel and counting.

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- 25 While the present invention has been described
in terms of the preferred embodiments, it is understood
that variations and modifications will occur to those
skilled in the art. Therefore, it is intended that the
appended claims cover all such equivalent variations
30 which come within the scope of the invention as claimed.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Weinstein, Jasminder
- (ii) TITLE OF INVENTION: Novel Mammalian Cell Cycle Protein
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Amgen Inc./Patent Operations/RBW
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 - (C) CITY: Thousand Oaks
 - (D) STATE: California
 - (E) COUNTRY: USA
 - (F) ZIP: 91320-1789
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1767 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown
- (ii) MOLECULE TYPE: cDNA
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 193..1692
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GGTAGACGGG CC ATG GCG CAG TTC GTG TTC GAG AGC GAT TTG CAT TCA	228
Met Ala Gln Phe Val Phe Glu Ser Asp Leu His Ser	
1 5 10	

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CGG GTC CTG AGT CTC ACC ATG AGT CCA GAC GGG GCC ACA GTG GCA TCT Arg Val Leu Ser Leu Thr Met Ser Pro Asp Gly Ala Thr Val Ala Ser 445 450 455 460	1572

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 465 470 475

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 Pro Ala Leu Arg Arg Glu Arg Glu Lys Ala Ser Thr Ser Lys Ser Ser
 480 485 490

CTC ATC CAC CAA GGC ATC CGG TGAAAGACAA CCCTTTCTTT TCCCTTCTTG 1719
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 495 500

ATTTTGTGTG TGTTTATTTT TTTCTAATAA AGTTCATATC TTCCTTTC 1767

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 499 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Gln Phe Val Phe Glu Ser Asp Leu His Ser Leu Leu Gln Leu
 1 5 10 15

Asp Ala Pro Ile Pro Asn Ala Pro Ile Ala Arg Trp Gln Arg Lys Ala
 20 25 30

Lys Glu Ala Thr Gly Pro Ala Pro Ser Pro Met Arg Ala Ala Asn Arg
 35 40 45

Ser His Ser Ala Gly Arg Thr Pro Gly Arg Thr Pro Gly Lys Ser Asn
 50 55 60

Ser Lys Val Gln Thr Thr Pro Ser Lys Pro Gly Gly Glu Arg Tyr Ile
 65 70 75 80

Pro Gln Arg Ser Ala Ser Gln Met Glu Val Ala Ser Phe Leu Leu Ser
 85 90 95

Lys Glu Asn Gln Pro Glu Asp Gly Gly Thr Pro Thr Lys Lys Glu His
 100 105 110

Gln Lys Ala Trp Ala Arg Asn Leu Asn Gly Phe Asp Val Glu Glu Ala
 115 120 125

Lys Ile Leu Arg Leu Ser Gly Lys Pro Gln Asn Ala Pro Glu Gly Tyr
 130 135 140

Gln Asn Arg Leu Lys Val Leu Tyr Ser Gln Lys Ala Thr Pro Gly Ser
 145 150 155 160

Ser Arg Lys Ala Cys Arg Tyr Ile Pro Ser Leu Pro Asp Arg Ile Leu
 165 170 175

- 55 -

Asp Ala Pro Glu Ile Arg Asn Asp Tyr Tyr Leu Asn Leu Val Asp Trp
 180 185 190
 Ser Ser Gly Asn Val Leu Ala Val Ala Leu Asp Asn Ser Val Tyr Leu
 195 200 205
 Trp Asn Ala Gly Ser Gly Asp Ile Leu Gln Leu Leu Gln Met Glu Gln
 210 215 220
 Pro Gly Asp Tyr Ile Ser Ser Val Ala Trp Ile Lys Glu Gly Asn Tyr
 225 230 235 240
 Leu Ala Val Gly Thr Ser Asn Ala Glu Val Gln Leu Trp Asp Val Gln
 245 250 255
 Gln Gln Lys Arg Leu Arg Asn Met Thr Ser His Ser Ala Arg Val Ser
 260 265 270
 Ser Leu Ser Trp Asn Ser Tyr Ile Leu Ser Ser Gly Ser Arg Ser Gly
 275 280 285
 His Ile His His His Asp Val Arg Val Ala Glu His His Val Ala Thr
 290 295 300
 Leu Ser Gly His Ser Gln Glu Val Cys Gly Leu Arg Trp Ala Pro Asp
 305 310 315 320
 Gly Arg His Leu Ala Ser Gly Gly Asn Asp Asn Ile Val Asn Val Trp
 325 330 335
 Pro Ser Gly Pro Gly Glu Ser Gly Trp Val Pro Leu Gln Thr Phe Thr
 340 345 350
 Gln His Gln Gly Ala Val Lys Ala Val Ala Trp Cys Pro Trp Gln Ser
 355 360 365
 Asn Ile Leu Ala Thr Gly Gly Gly Thr Ser Asp Arg His Ile Arg Ile
 370 375 380
 Trp Asn Val Cys Ser Gly Ala Cys Leu Ser Ala Val Asp Val His Ser
 385 390 395 400
 Gln Val Cys Ser Ile Leu Trp Ser Pro His Tyr Lys Glu Leu Ile Ser
 405 410 415
 Gly His Gly Phe Ala Gln Asn Gln Leu Val Ile Trp Lys Tyr Pro Thr
 420 425 430
 Met Ala Lys Val Ala Glu Leu Lys Gly His Thr Ala Arg Val Leu Ser
 435 440 445
 Leu Thr Met Ser Pro Asp Gly Ala Thr Val Ala Ser Ala Ala Asp
 450 455 460
 Glu Thr Leu Arg Leu Trp Arg Cys Phe Glu Leu Asp Pro Ala Leu Arg
 465 470 475 480

- 56 -

Arg Glu Arg Glu Lys Ala Ser Thr Ser Lys Ser Ser Leu Ile His Gln
 485 490 495

Gly Ile Arg

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1700 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 117..1616

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTCGACCCAC GCGTCCGGGC GTAAGCCAGG CGTGTTAAAG CCGGTCGGAA CTGCTCCGGA	60
GGGCACGGGC TCCGTAGGCA CCAACTGCAA GGACCCCTCC CCCTGCGGGC GCTCCC	116
ATG GCA CAG TTC GCG TTC GAG AGT GAC CTG CAC TCG CTG CTT CAG CTG	164
Met Ala Gln Phe Ala Phe Glu Ser Asp Leu His Ser Leu Leu Gln Leu	
1 5 10 15	
GAT GCA CCC ATC CCC AAT GCA CCC CCT GCG CGC TGG CAG CGC AAA GCC	212
Asp Ala Pro Ile Pro Asn Ala Pro Pro Ala Arg Trp Gln Arg Lys Ala	
20 25 30	
AAG GAA GCC GCA GGC CCG GCC CCC TCA CCC ATG CGG GCC GCC AAC CGA	260
Lys Glu Ala Ala Gly Pro Ala Pro Ser Pro Met Arg Ala Ala Asn Arg	
35 40 45	
TCC CAC AGC GCC GGC AGG ACT CCG GGC CGA ACT CCT GGC AAA TCC AGT	308
Ser His Ser Ala Gly Arg Thr Pro Gly Arg Thr Pro Gly Lys Ser Ser	
50 55 60	
TCC AAG GTT CAG ACC ACT CCT AGC AAA CCT GGC GGT GAC CGC TAT ATC	356
Ser Lys Val Gln Thr Thr Pro Ser Lys Pro Gly Gly Asp Arg Tyr Ile	
65 70 75 80	
CCC CAT CGC AGT GCT GCC CAG ATG GAG GTG GCC AGC TTC CTC CTG AGC	404
Pro His Arg Ser Ala Ala Gln Met Glu Val Ala Ser Phe Leu Leu Ser	
85 90 95	
AAG GAG AAC CAG TCT GAA AAC AGC CAG ACG CCC ACC AAG AAG GAA CAT	452
Lys Glu Asn Gln Ser Glu Asn Ser Gln Thr Pro Thr Lys Lys Glu His	
100 105 110	

- 57 -

CAG AAA GCC TGG GCT TTG AAC CTG AAC GGT TTT GAT GTA GAG GAA GCC Gln Lys Ala Trp Ala Leu Asn Leu Asn Gly Phe Asp Val Glu Glu Ala 115 120 125	500
AAG ATC CTT CGG CTC AGT GGA AAA CCA CAA AAT GCG CCA GAG GGT TAT Lys Ile Leu Arg Leu Ser Gly Lys Pro Gln Asn Ala Pro Glu Gly Tyr 130 135 140	548
CAG AAC AGA CTG AAA GTA CTC TAC AGC CAA AAG GCC ACT CCT GGC TCC Gln Asn Arg Leu Lys Val Leu Tyr Ser Gln Lys Ala Thr Pro Gly Ser 145 150 155 160	596
AGC CGG AAG ACC TGC CGT TAC ATT CCT TCC CTG CCA GAC CGT ATC CTG Ser Arg Lys Thr Cys Arg Tyr Ile Pro Ser Leu Pro Asp Arg Ile Leu 165 170 175	644
GAT GCG CCT GAA ATC CGA AAT GAC TAT TAC CTG AAC CTT GTG GAT TGG Asp Ala Pro Glu Ile Arg Asn Asp Tyr Tyr Leu Asn Leu Val Asp Trp 180 185 190	692
AGT TCT GGG AAT GTA CTG GCC GTG GCA CTG GAC AAC AGT GTG TAC CTG Ser Ser Gly Asn Val Leu Ala Val Ala Leu Asp Asn Ser Val Tyr Leu 195 200 205	740
TGG AGT GCA AGC TCT GGT GAC ATC CTG CAG CTT TTG CAA ATG GAG CAG Trp Ser Ala Ser Ser Gly Asp Ile Leu Gln Leu Leu Gln Met Glu Gln 210 215 220	788
CCT GGG GAA TAT ATA TCC TCT GTG GCC TGG ATC AAA GAG GGC AAC TAC Pro Gly Glu Tyr Ile Ser Ser Val Ala Trp Ile Lys Glu Gly Asn Tyr 225 230 235 240	836
TTG GCT GTG GGC ACC AGC AGT GCT GAG GTG CAG CTA TGG GAT GTG CAG Leu Ala Val Gly Thr Ser Ser Ala Glu Val Gln Leu Trp Asp Val Gln 245 250 255	884
CAG CAG AAA CGG CTT CGA AAT ATG ACC AGT CAC TCT GCC CGA GTG GGC Gln Gln Lys Arg Leu Arg Asn Met Thr Ser His Ser Ala Arg Val Gly 260 265 270	932
TCC CTA AGC TGG AAC AGC TAT ATC CTG TCC AGT GGT TCA CGT TCT GGC Ser Leu Ser Trp Asn Ser Tyr Ile Leu Ser Ser Gly Ser Arg Ser Gly 275 280 285	980
CAC ATC CAC CAC CAT GAT GTT CGG GTA GCA GAA CAC CAT GTG GCC ACA His Ile His His His Asp Val Arg Val Ala Glu His His Val Ala Thr 290 295 300	1028
CTG AGT GGC CAC AGC CAG GAA GTG TGT GGG CTG CGC TGG GCC CCA GAT Leu Ser Gly His Ser Gln Glu Val Cys Gly Leu Arg Trp Ala Pro Asp 305 310 315 320	1076
GGA CGA CAT TTG GCC AGT GGT GGT AAT GAT AAC TTG GTC AAT GTG TGG Gly Arg His Leu Ala Ser Gly Gly Asn Asp Asn Leu Val Asn Val Trp 325 330 335	1124

- 58 -

CCT AGT GCT CCT GGA GAG GGT GGC TGG GTT CCT CTG CAG ACA TTC ACC Pro Ser Ala Pro Gly Glu Gly Gly Trp Val Pro Leu Gln Thr Phe Thr 340 345 350	1172
CAG CAT CAA GGG GCT GTC AAG GCC GTA GCA TGG TGT CCC TGG CAG TCC Gln His Gln Gly Ala Val Lys Ala Val Ala Trp Cys Pro Trp Gln Ser 355 360 365	1220
AAT GTC CTG GCA ACA GGA GGG GGC ACC AGT GAT CGA CAC ATT CGC ATC Asn Val Leu Ala Thr Gly Gly Gly Thr Ser Asp Arg His Ile Arg Ile 370 375 380	1268
TGG AAT GTG TGC TCT GGG GCC TGT CTG AGT GCC GTG GAT GCC CAT TCC Trp Asn Val Cys Ser Gly Ala Cys Leu Ser Ala Val Asp Ala His Ser 385 390 395 400	1316
CAG GTG TGC TCC ATC CTC TGG TCT CCC CAT TAC AAG GAG CTC ATC TCA Gln Val Cys Ser Ile Leu Trp Ser Pro His Tyr Lys Glu Leu Ile Ser 405 410 415	1364
GGC CAT GGC TTT GCA CAG AAC CAG CTA GTT ATT TGG AAG TAC CCA ACC Gly His Gly Phe Ala Gln Asn Gln Leu Val Ile Trp Lys Tyr Pro Thr 420 425 430	1412
ATG GCC AAG GTG GCT GAA CTC AAA GGT CAC ACA TCC CGG GTC CTG AGT Met Ala Lys Val Ala Glu Leu Lys Gly His Thr Ser Arg Val Leu Ser 435 440 445	1460
CTG ACC ATG AGC CCA GAT GGG GCC ACA GTG GCA TCC GCA GCA GCA GAT Leu Thr Met Ser Pro Asp Gly Ala Thr Val Ala Ser Ala Ala Ala Asp 450 455 460	1508
GAG ACC CTG AGG CTA TGG CGC TGT TTT GAG TTG GAC CCT GCG CGG CGG Glu Thr Leu Arg Leu Trp Arg Cys Phe Glu Leu Asp Pro Ala Arg Arg 465 470 475 480	1556
CGG GAG CGG GAG AAG GCC AGT GCA GCC AAA AGC AGC CTC ATC CAC CAA Arg Glu Arg Glu Lys Ala Ser Ala Ala Lys Ser Ser Leu Ile His Gln 485 490 495	1604
GGC ATC CGC TGAAGACCAA CCCATCACCT CAGTTGTTTT TTATTTTTCT Gly Ile Arg 500	1653
AATAAAGTCA TGTCTCCCTT CATGTTTTTT TTTTAAAAA AAAAAA	1700

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 499 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ala Gln Phe Ala Phe Glu Ser Asp Leu His Ser Leu Leu Gln Leu
 1 5 10 15
 Asp Ala Pro Ile Pro Asn Ala Pro Pro Ala Arg Trp Gln Arg Lys Ala
 20 25 30
 Lys Glu Ala Ala Gly Pro Ala Pro Ser Pro Met Arg Ala Ala Asn Arg
 35 40 45
 Ser His Ser Ala Gly Arg Thr Pro Gly Arg Thr Pro Gly Lys Ser Ser
 50 55 60
 Ser Lys Val Gln Thr Thr Pro Ser Lys Pro Gly Gly Asp Arg Tyr Ile
 65 70 75 80
 Pro His Arg Ser Ala Ala Gln Met Glu Val Ala Ser Phe Leu Leu Ser
 85 90 95
 Lys Glu Asn Gln Ser Glu Asn Ser Gln Thr Pro Thr Lys Lys Glu His
 100 105 110
 Gln Lys Ala Trp Ala Leu Asn Leu Asn Gly Phe Asp Val Glu Glu Ala
 115 120 125
 Lys Ile Leu Arg Leu Ser Gly Lys Pro Gln Asn Ala Pro Glu Gly Tyr
 130 135 140
 Gln Asn Arg Leu Lys Val Leu Tyr Ser Gln Lys Ala Thr Pro Gly Ser
 145 150 155 160
 Ser Arg Lys Thr Cys Arg Tyr Ile Pro Ser Leu Pro Asp Arg Ile Leu
 165 170 175
 Asp Ala Pro Glu Ile Arg Asn Asp Tyr Tyr Leu Asn Leu Val Asp Trp
 180 185 190
 Ser Ser Gly Asn Val Leu Ala Val Ala Leu Asp Asn Ser Val Tyr Leu
 195 200 205
 Trp Ser Ala Ser Ser Gly Asp Ile Leu Gln Leu Leu Gln Met Glu Gln
 210 215 220
 Pro Gly Glu Tyr Ile Ser Ser Val Ala Trp Ile Lys Glu Gly Asn Tyr
 225 230 235 240
 Leu Ala Val Gly Thr Ser Ser Ala Glu Val Gln Leu Trp Asp Val Gln
 245 250 255
 Gln Gln Lys Arg Leu Arg Asn Met Thr Ser His Ser Ala Arg Val Gly
 260 265 270
 Ser Leu Ser Trp Asn Ser Tyr Ile Leu Ser Ser Gly Ser Arg Ser Gly
 275 280 285
 His Ile His His His Asp Val Arg Val Ala Glu His His Val Ala Thr
 290 295 300

- 60 -

Leu Ser Gly His Ser Gln Glu Val Cys Gly Leu Arg Trp Ala Pro Asp
 305 310 315 320
 Gly Arg His Leu Ala Ser Gly Gly Asn Asp Asn Leu Val Asn Val Trp
 325 330 335
 Pro Ser Ala Pro Gly Glu Gly Gly Trp Val Pro Leu Gln Thr Phe Thr
 340 345 350
 Gln His Gln Gly Ala Val Lys Ala Val Ala Trp Cys Pro Trp Gln Ser
 355 360 365
 Asn Val Leu Ala Thr Gly Gly Gly Thr Ser Asp Arg His Ile Arg Ile
 370 375 380
 Trp Asn Val Cys Ser Gly Ala Cys Leu Ser Ala Val Asp Ala His Ser
 385 390 395 400
 Gln Val Cys Ser Ile Leu Trp Ser Pro His Tyr Lys Glu Leu Ile Ser
 405 410 415
 Gly His Gly Phe Ala Gln Asn Gln Leu Val Ile Trp Lys Tyr Pro Thr
 420 425 430
 Met Ala Lys Val Ala Glu Leu Lys Gly His Thr Ser Arg Val Leu Ser
 435 440 445
 Leu Thr Met Ser Pro Asp Gly Ala Thr Val Ala Ser Ala Ala Ala Asp
 450 455 460
 Glu Thr Leu Arg Leu Trp Arg Cys Phe Glu Leu Asp Pro Ala Arg Arg
 465 470 475 480
 Arg Glu Arg Glu Lys Ala Ser Ala Ala Lys Ser Ser Leu Ile His Gln
 485 490 495
 Gly Ile Arg

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WHAT IS CLAIMED IS:

1. An isolated DNA encoding a biologically active p55CDC polypeptide selected from the group consisting of:
 - a) DNA having a nucleotide sequence encoding the amino acid sequence of SEQ. ID. NO: 2;
 - b) DNA having a nucleotide sequence encoding the amino acid sequence of SEQ. ID. NO: 4; and
 - 10 c) DNA having a nucleotide sequence which hybridizes with the DNA of (a) or (b), or with a fragment thereof, wherein the hybridizing DNA encodes a polypeptide having the biological activity of p55CDC.
- 15 2. An isolated DNA of Claim 1 which is cDNA, genomic DNA or synthetic DNA.
3. An isolated DNA of Claim 1 which includes one or more codons preferred for expression in *E. coli*
20 host cells.
4. A biologically functional plasmid or viral DNA vector including DNA of Claim 1.
- 25 5. A procaryotic or eucaryotic host cell stably transformed or transfected with a DNA vector of Claim 4.
6. An isolated p55CDC polypeptide.
- 30 7. A polypeptide of Claim 6 having the amino acid sequence of SEQ. ID. NO: 4.
8. A polypeptide of Claim 6 characterized by
35 being the product of procaryotic or eucaryotic expression of an exogenous DNA sequence.

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9. A polypeptide having an amino acid sequence encoded by the DNA of Claim 1.

5 10. A polypeptide according to Claim 9 having the ability of forming a complex having cell-cycle dependent kinase activity.

11. A method of producing a p55CDC
10 polypeptide comprising culturing the host cell of Claim 5 to allow the host cell to express the p55CDC polypeptide.

12. An antibody specifically binding a
15 polypeptide encoded by the DNA of Claim 1.

13. An antibody of Claim 12 which is a monoclonal antibody.

20 14. A complex of two or more proteins wherein one of the proteins is p55CDC and wherein the complex has cell cycle dependent kinase activity.

15. A method of modulating cell division
25 comprising introducing into a cell a compound which modulates the cell cycle-dependent kinase activity of the complex of Claim 14.

16. A method according to Claim 15 wherein
30 the compound is selected from the group consisting of:
a) compounds which increase or decrease the levels of p55CDC synthesis;
b) compounds which interfere with the
formation of a p55CDC complex having cell cycle
35 dependent kinase activity; and

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c) compounds which promote the formation of a p55CDC complex or stabilize the complex.

17. A method according to Claim 16 wherein
5 the compounds are nucleic acid molecules, polypeptides, peptides, antibodies, carbohydrates and organic molecules.

18. A method according to Claim 15 wherein
10 cell division is inhibited by introducing into a cell a compound in an amount sufficient to inhibit the cell cycle-dependent kinase activity.

19. A method according to Claim 18 wherein
15 the compound is selected from the group consisting of:
a) compounds which decrease the levels of p55CDC synthesis; and
b) compounds which interfere with the
formation of a p55CDC complex having cell cycle
20 dependent kinase activity.

20. A method according to Claim 18 wherein
the cell is a tumor cell.

21. A method of chemotherapy comprising
25 treating a mammal with a compound in an amount sufficient to inhibit the kinase activity of the complex of Claim 14 in a pharmaceutically effective adjuvant.

22. A method for detecting the level of
30 p55CDC in biological fluids comprising the steps of incubating an antibody specific for p55CDC with said fluid under conditions suitable for forming a complex between the antibody and p55CDC and detecting the
35 presence of the antibody-p55CDC complex.

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23. A method for detecting the level of a complex according to Claim 14 in biological fluids comprising the steps of incubating an antibody specific for p55CDC with said fluid under conditions suitable for antibody binding to p55CDC and detecting the presence of antibody bound to the complex.
- 5

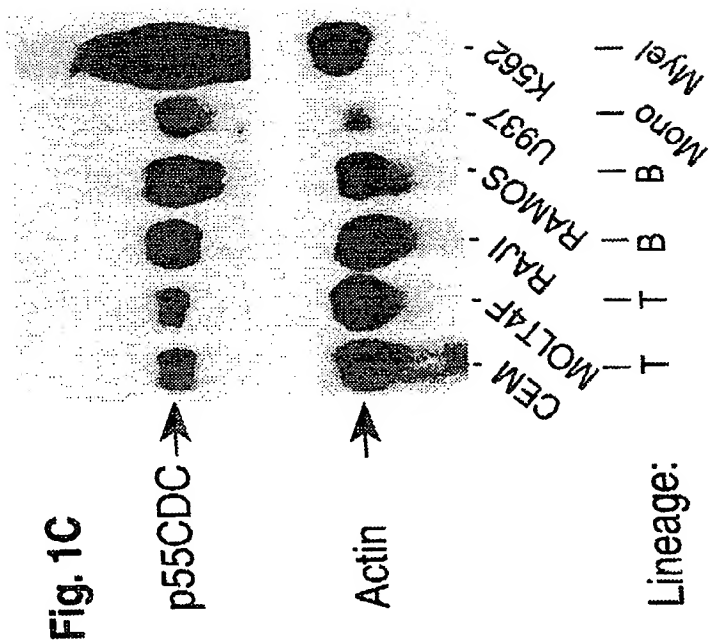
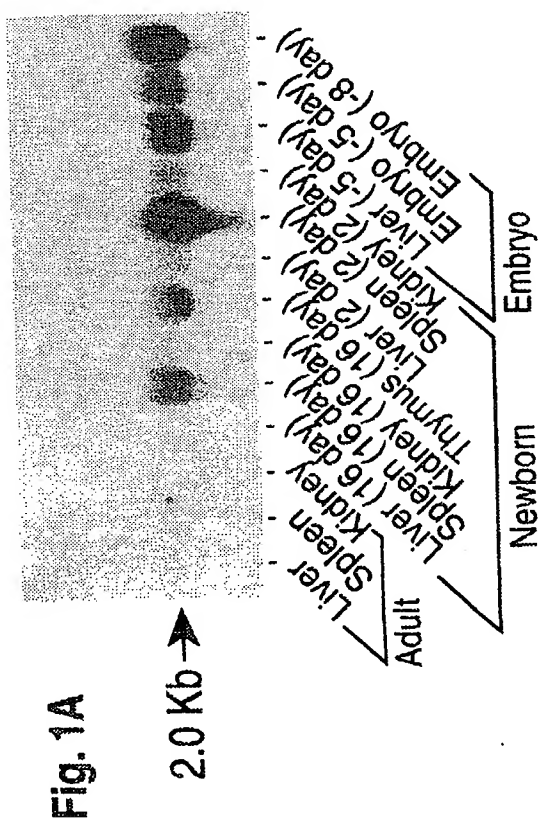
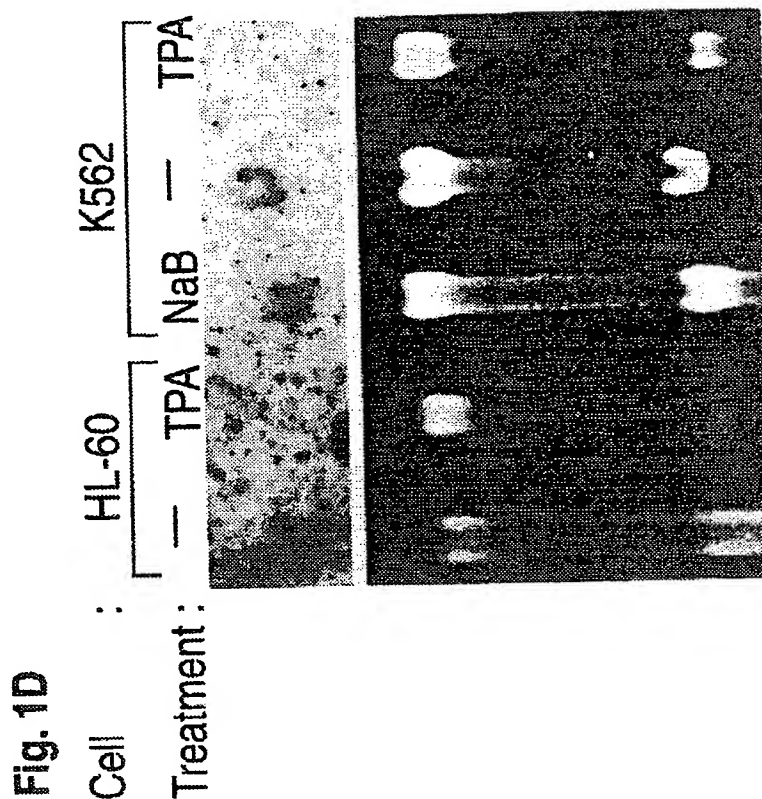
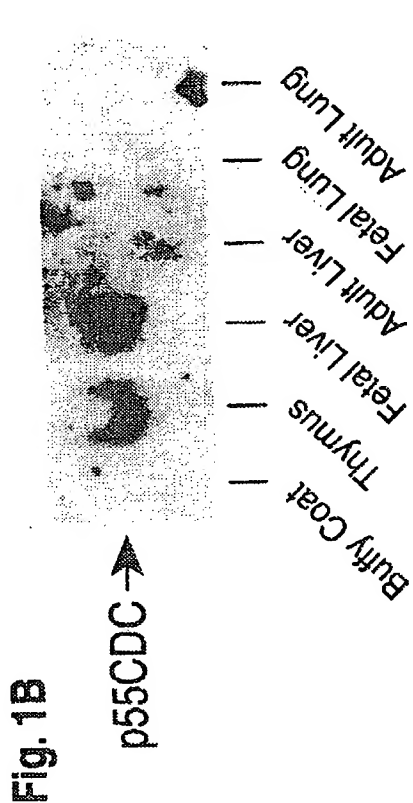


Fig. 2A

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1      G G C G T G C C T T T A G C C G G T C A G A A A G A C G C A T T C G G C A C T T C T A C A G C C A C T C A G G A G T C A G G A T T T G
73 T G T T T G C G A G A G G T T A C G A A G A G G T G C T G G C T G G T G C G A A C T G T G G C A G A G C C C A G A G T C C T G C G A G G T C C T G A G T T T G T G C G C T C T C A C C C C C C C G G T A G A C G G G C C
      A      C      T      C      C      G      G      T      A      C C C      G      C      G      G      G
193 A T G G C G C A G T T C G T G T T C G A G A G C G A T T G C A T T G C A C T G G A G C G C C C A T C C C C A T G C A C C G A T T G C T G C G T G G C A G C G G A A A G C A A A G A G C C A C A G G C C C A G C C C C C
      M A Q F V F E S D L H S L L Q L D A P I P N A P I A R W Q R K A K E A T G P A P 40
      A
      A      C      C      C      C A      T      C G      C      T      C      C      C
313 T G G C C T A T G C G G C C G C C A C A G A T C A C A G C C G G T C G G A C C C C G G C C A C T C C T G G C A A T C T A A T T C T A A G T T C A G A C C C C C T A G C A A C C T G G A G G T G A G C G C C T A T C
      S P M R A A N R S H S A G R T P G R T P G K S N S K V Q T T P S K P G G E R Y I 80
      S
      T      C      G      G      C      T      T      A      A      C C A G      A      T T
433 C C C C A G C G T A G T T C C C A A T G G A G G T G G C C A G C T T C C T T T G A G C A G G A G A C C A G C C G G A G A G C G G G T A C C C C A G A G A G G A G C A T C A G A A G C C T G G G C T C G G A C C T G
      P Q R S A S Q M E V A S F L L S K E N Q P E D G G T P T K K E H Q K A W A R N L 120
      H      A      S      N      S      Q      L

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SUBSTITUTE SHEET (RULE 26)

Fig. 2B

A TC A A G G T T C A G T
 553 AACGGTTTGTGAGGAGCCAGATCCTCAGGCTCAGTGGAAACCTCAGATGCCCCAGAGGCTACGAGACAGATTGAAGTACTCTACAGCCAGAAAGCCAGCCGCTGCTCC
 N G F D V E E A K I L R L S G K P Q N A P E G Y Q N R L K V L Y S Q K A T P G S 160

C A C C T C T C G G A T C G T G C G T G C G C
 673 AGTGGGAAGGCTTGCAGATACATTCCTCCCTGCCAGACAGGATTCCTGATGCCCTGAAATCCGGAATGACTACTACCTGATCTGCGATTGGAGCTCTGGAATGATTAGCTGTG
 S R K A C R Y I P S L P D R I L D A P E I R N D Y Y L N L V D W S S G N V L A V 200
 T

C G G T A A C T T A T C T
 793 GCACTGGACACAGTGTGTAATGGAACGCTGGTCCGGTACATCCCTGCAGCTGTGCAATGGAGAGCCCTGGGACTACATACTATCCGTGGCTGGATCAAGAGGGCAACTAC
 A L D N S V Y L W N A G S G D I L Q L L Q M E Q P G D Y I S S V A W I K E G N Y 240
 S S E

T C G T T C G G A C
 913 CTGGCTGTGGCACCAGTAATGCTGAGGTGAGCTATGGGATGTGCAGCAGCAGAAAGGGCTTCGAAACATGACCAGCCACTCTGCTCGAGTAAGCTCCTGAGTTGGAACAGCTATAC
 L A V G T S N A E V Q L W D V Q Q K R L R N M T S H S A R V S S L S W N S Y I 280
 S G

SUBSTITUTE SHEET (RULE 26)

Fig. 2C

A TC A A G G I T C A G T
 1033 AACGGTTTGTGAGGAGCCCAAGATCCTCAGGCTCAGTGGAAACCTCAGATGCCCCAGAGGCTACCAGAACAGATTGAAGTACTCTACAGCCAGCAAGCCAGGCTGGCTCC
 N G F D V E E A K I L R L S G K P Q N A P E G Y Q N R L K V L Y S Q K A T P G S 320

T C T I T G T C G G T C G G C
 1153 GGACGACATCIGGCAAGCGGTGGCAATGATAACATGTCAACGTGTGGCTAGTGGTCTGGAGAAAGTGGCTGGGTCCCTGGCAGACATTCACTCAACATCAAGGTGTCTCAGGCT
 G R H L A S G G N D N I V N V W P S G P G E S G W V P L Q T F T Q H Q G A V K A 360
 L A G

A G G C T C T G G C C
 1273 GTTGCATGGTCCCTGGCAGTCCATATCCTGGCAACAGGAGGTACCGAGTGGACGACATTCGATTGGACGCTCIGCTCGGAGGCTGTCTGAGTGTGGAATGCGATTCC
 V A W C P W Q S N I L A T G G G T S D R H I R I W N V C S G A C L S A V D V H S 400
 V A

T C A A T A
 1393 CAGGTGTGCTCCATCCTCIGGCTCCCGCACTATAAGGAGCTCATCTCAGGCCATGGCTTGGCCAGAACAGGCTGTTATTTGGAAGTACCAACCATGGCCAGGTGGCAGAGCTCAAA
 Q V C S I L W S P H Y K E L I S G H G F A Q N Q L V I W K Y P T M A K V A E L K 440

Fig. 3A

IPSLPDRILD	APEIRNDYYLNL	VDWS	SGNV	LAVALL	DNSVYLWN	(168-210)
AGSGD	ILQLLQMEQPGDYISS	VAWI	KEGNY	LAVGT	SNAEVQLWD	(211-254)
VQ	QQRRLR	NMTSHSARVSS	NSYI	LSSGSR	S GHIHHHD	(255-294)
VRVAE	HHV	ATLSGHSQEVCGLRWAP	DGRHL	ASGNGN	DNIVNVWP	(295-336)
SGPGESGW	PLQTFTHQHGAVKA	VAWCPWQSNILATGGTSDRHIRIWN				(337-386)
VCSGAC	LSA	VDVHSQ	VCS	ILWSP	HYKELISGHGFAQNQLVIWK	(387-429)
YPTIMAK	V	AE	LKGHTARVLS	LTMSP	DGATVASAA	ADETLR
						LWR (430-471)

Fig. 3B

CDC20	PERILDA	PGFQDDFYLNLL	SW KKNVLAIALDTAL	YLWN	(249-287)	
p55CDC	LPDRILDA	PEIRNDYYLNLV	DW SGNVLAVALDNSV	YLWS	(171-210)	
CDC4	LSDEIFSA	INNNLPHAYFKNLL	FRLVANMDR	ELSDLGTLIKDNLKRDLLITS	(226-277)	
CDC20	AT	TGDIVSLTDFENTT	ICSVT	WSD	(288-329)	
p55CDC	AS	SGDILQLLOMEQPGEY	ISSVA	WIK	(211-254)	
CDC4	LPFEISLKI	FNYLQFE	DIINSLGVSONWNKII	RKSTSLWKKLLISENFVSPKGFNSLNLKLSQKYPK	(278-344)	
CDC20	VEIMSLIR		TMRSGLGVIRIGLSWLDTIL	IA TGSRSGETIQIND	(330-370)	
p55CDC	VQQQKRLR		NMTS HSARVGSLSWNSYIL	SSGSRSGHIIHHHD	(255-294)	
CDC4	LSQQDRRL	LSFLENIFILKNWYNPKFVPQRTTLRGHMTS	VITCLOQFEDNYW	ITGADDDKMI	IRVYD	(345-408)
CDC20	VRIKQHI	IVSTWAEHTGEVCGLSYKSDGLQL	ASGGNDNTVM	IWD	(371-413)	
p55CDC	VRVAEHVAT	LSGHSQEVCGLRWAPDGRHL	ASGGNDNLVN	VMP	(295-336)	
CDC4	SINKKFL	LQLSGHDGCVWALKYAHGG	ILVSGSTDR	TVRVWD	(409-449)	

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Fig. 3C

CDC20	TR	TSLPQFSKKTHTTAAVKALSWCPYSPNINNSGGGQTDKHHFWN	(414-458)
p55CDC	SAPGEGGWVPLQTFTTQHQA VKAVAWCPWQSNVLATGGGTSDRHRIWN	(337-386)	
CDC4	IKKGCCTHV	FEGHNSTVRCLDIVEYKNIKYIVTGSRDNTLHVWK	(450-498)
CDC20	SITGARVGSINTGSQVSSLHW	(459-479)	
p55CDC	VCSGACLSA VDAHSQVC SILWSPH	(387-429)	
CDC4	LPKESSVPDHGEEHDYPLVFHTPEENPYFVGVLRGHMASVRTVSGHGNI VVSGSYDNTLIVWD	(499-556)	
p55CDC	YPTMAKV AELKGHTSRVLSLTMSPPDGATVASAADET LRLWR	(430-471)	
CDC4	VAQMCKCLYILSGHTDRIYSTIYDHERKRCSASMDDTIRIWD	(557-598)	

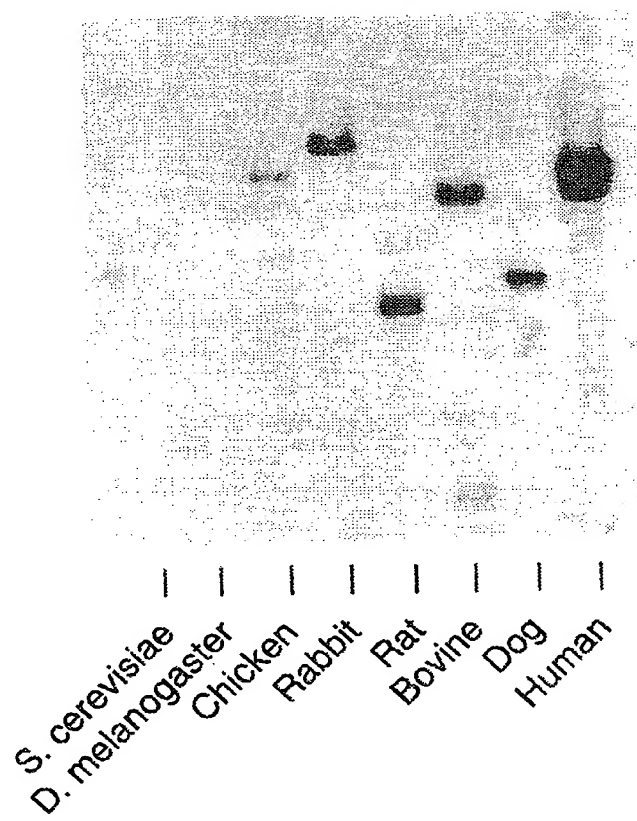
Fig. 4**SUBSTITUTE SHEET (RULE 26)**

Fig. 5A

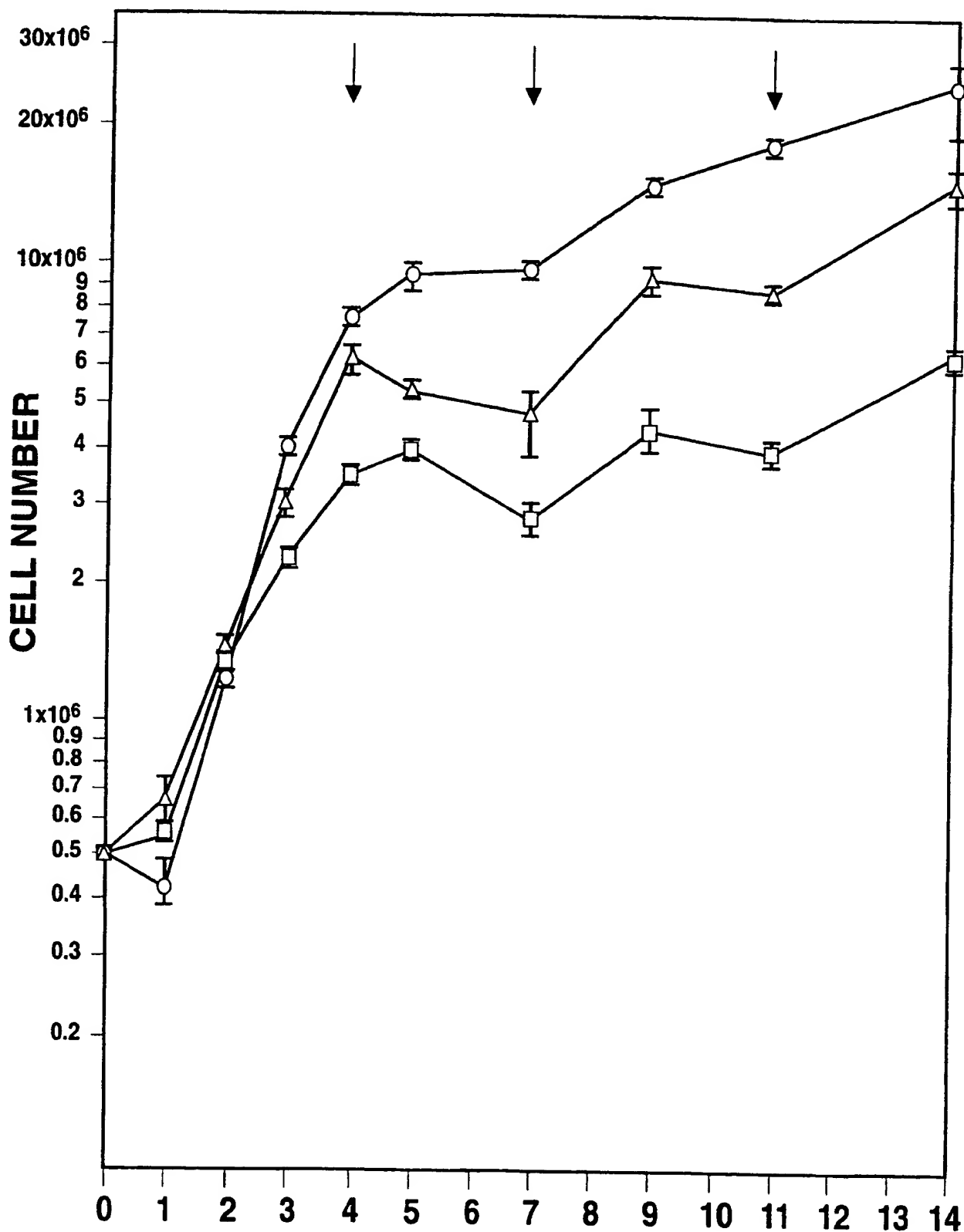


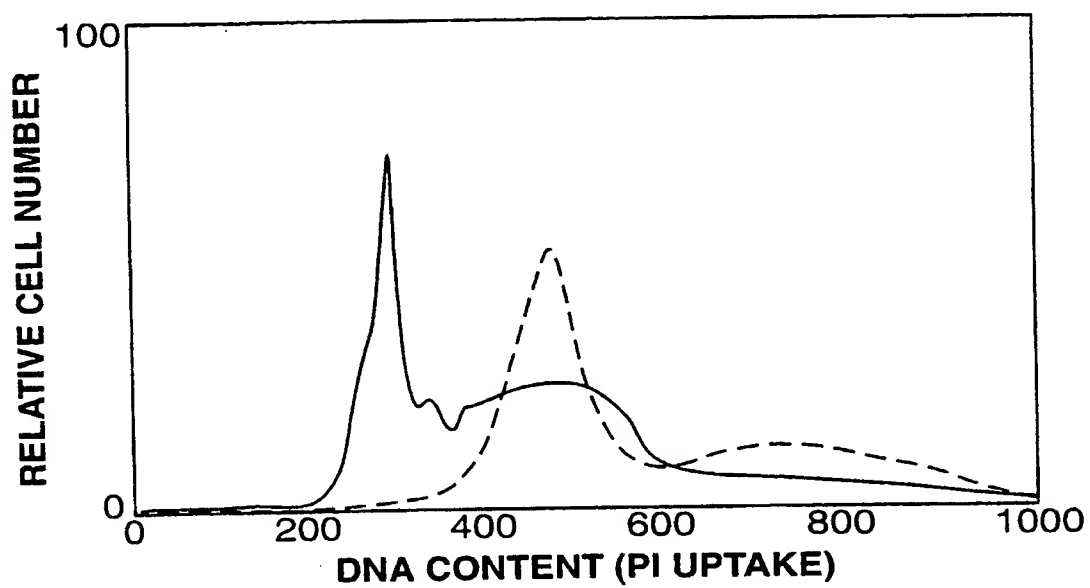
Fig. 5B

Fig. 6A

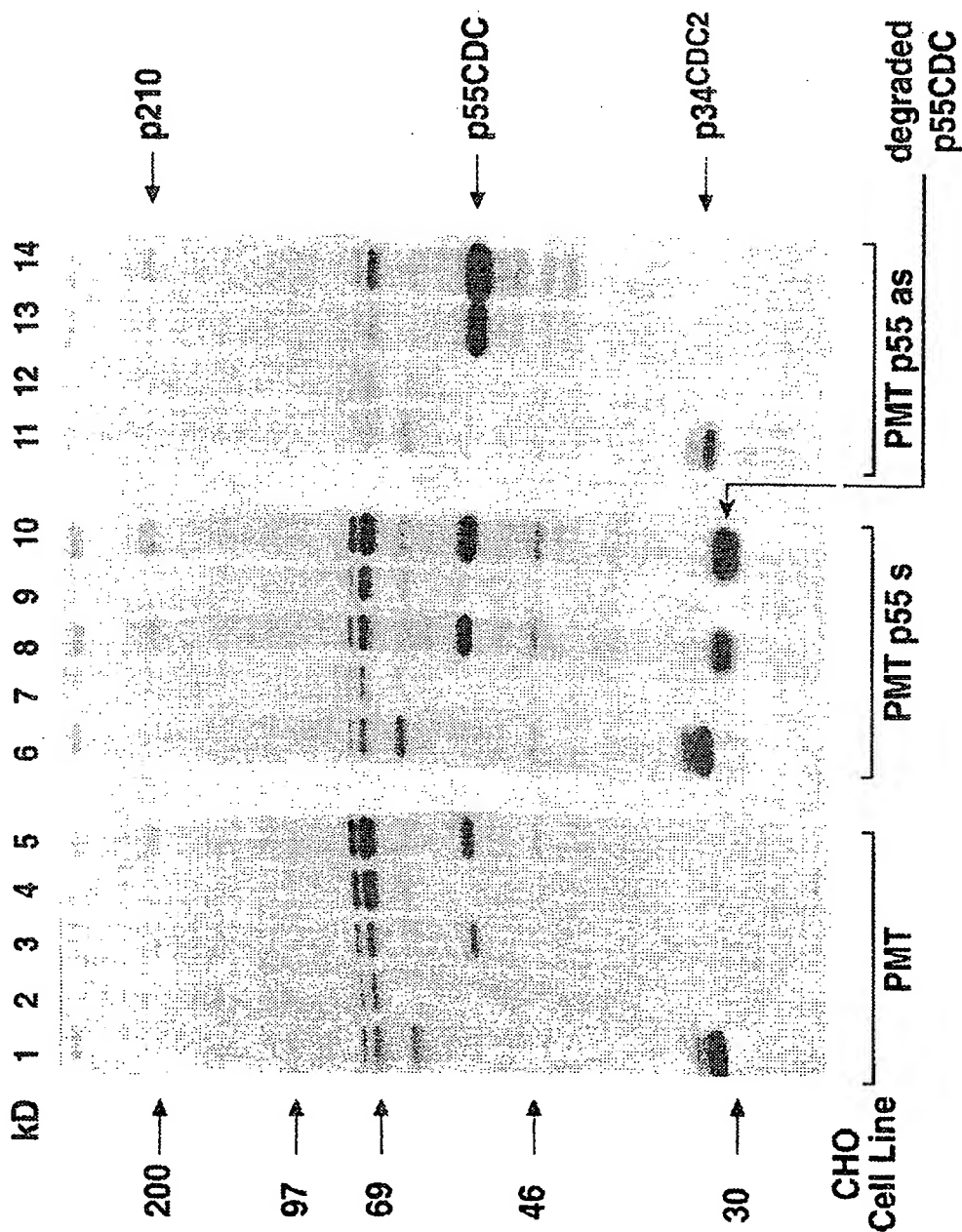


Fig. 6B

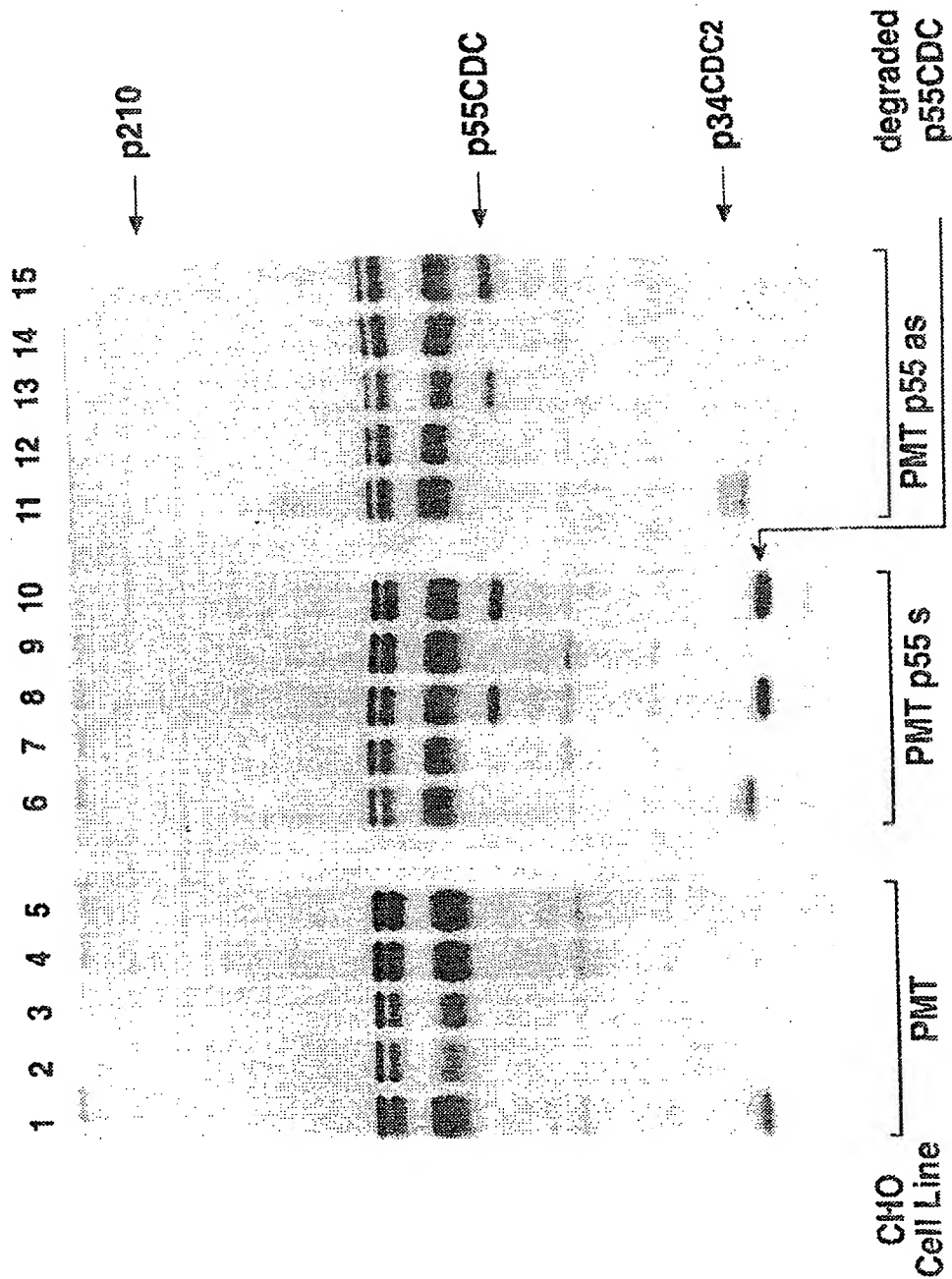


Fig. 7A

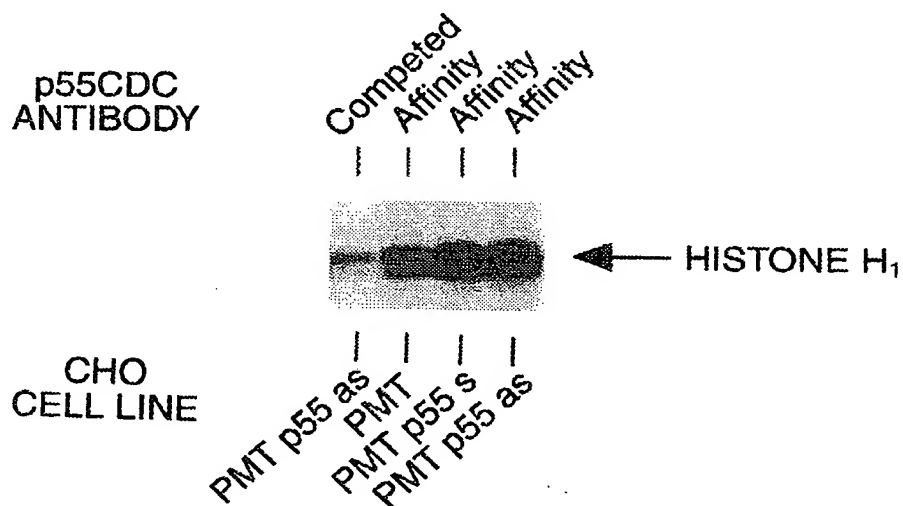


Fig. 7B

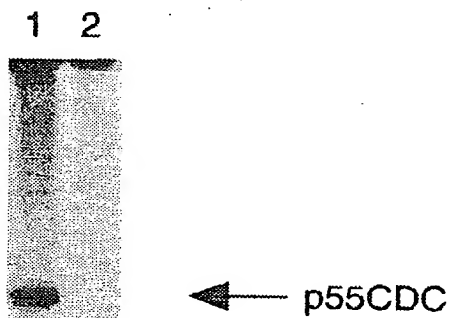
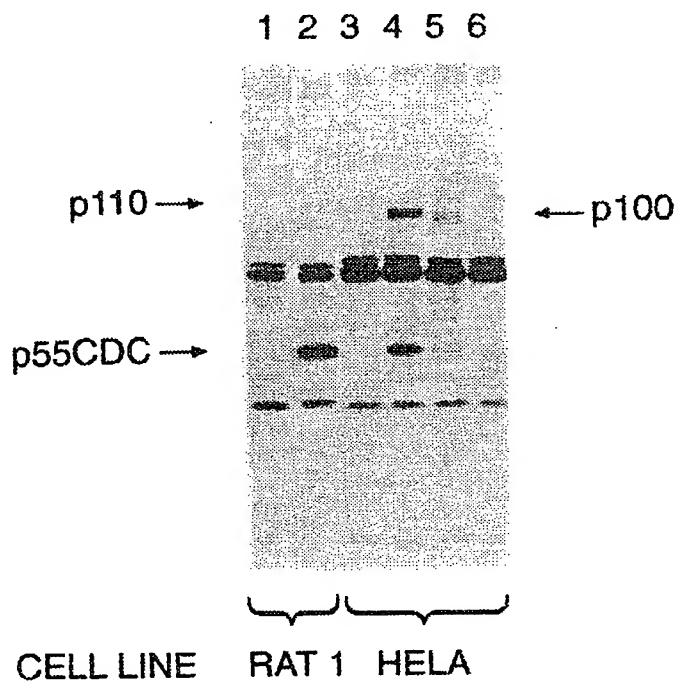
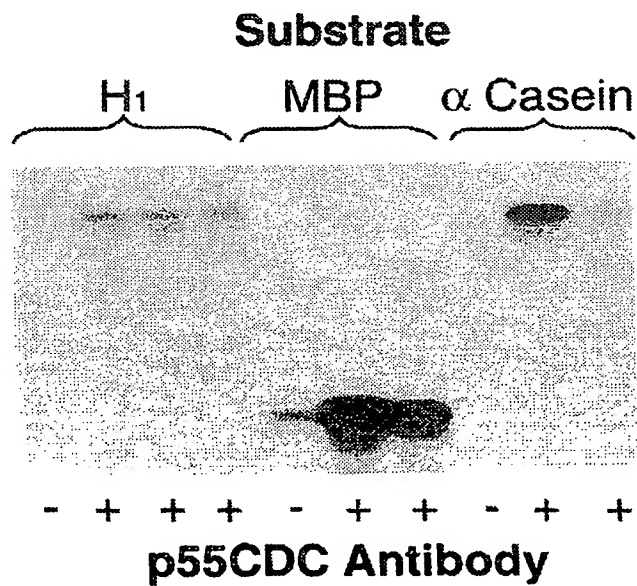


Fig. 8A**Fig. 8B**

SUBSTITUTE SHEET (RULE 26)

Fig. 8C

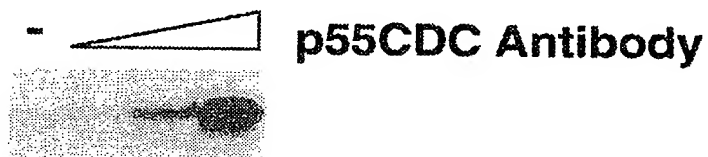


Fig. 8D

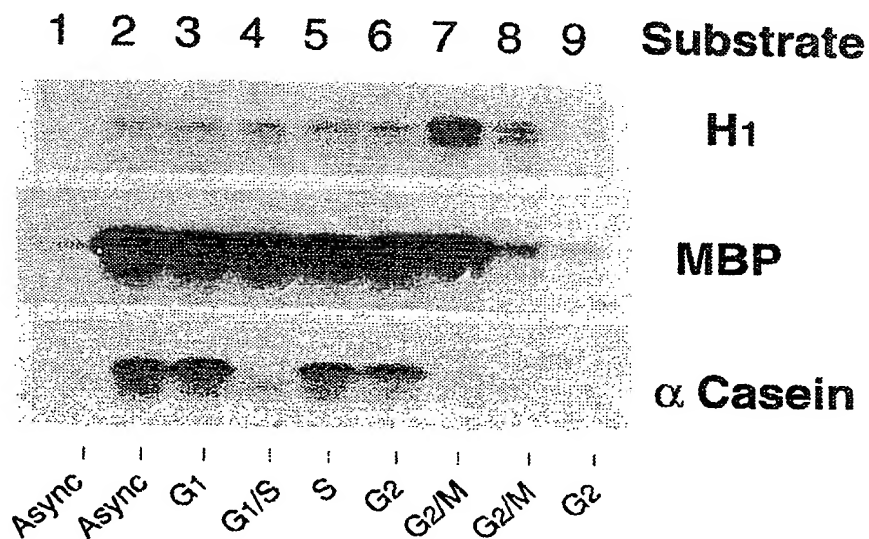


Fig. 8E

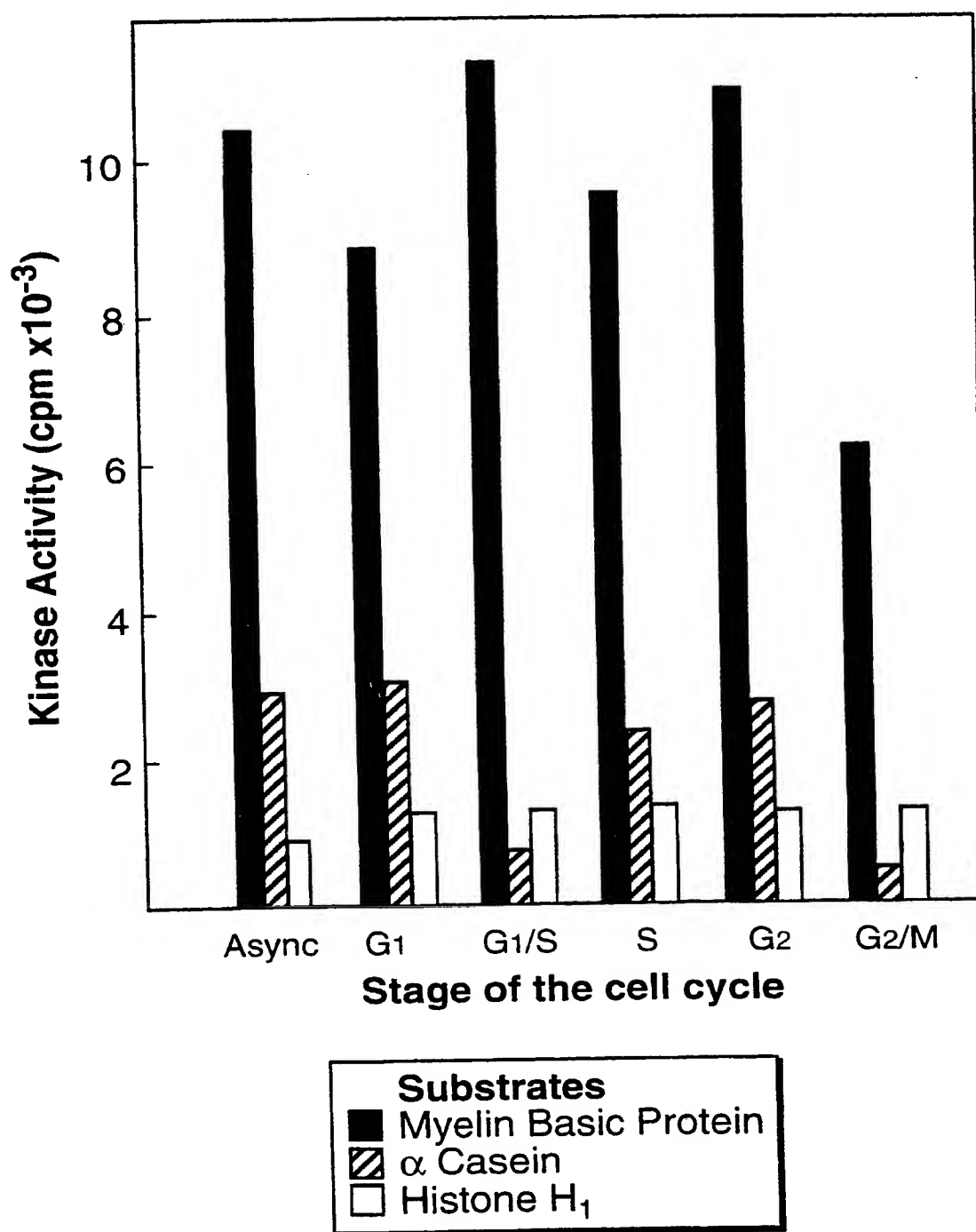


Fig. 9A

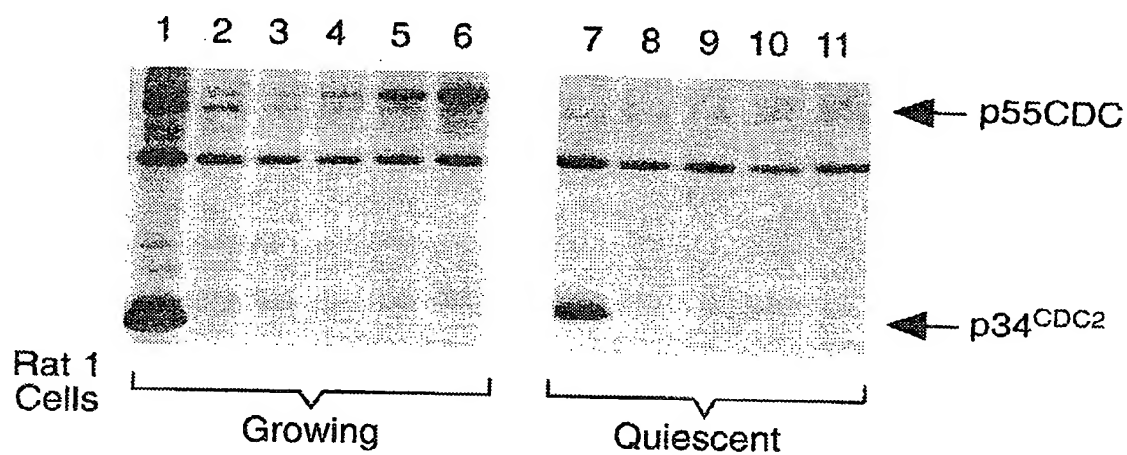
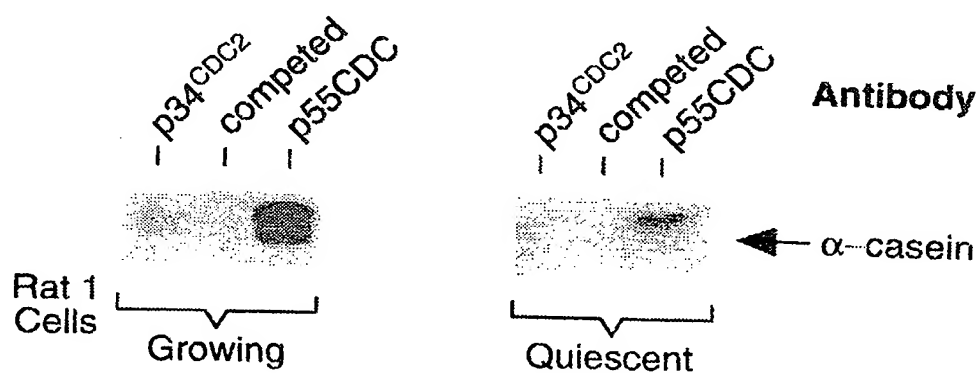


Fig. 9B



INTERNATIONAL SEARCH REPORT

Application No
PCT/US 95/01806

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/86 C12N1/21 C12N5/10 C07K14/47
C07K16/18 A61K47/42 A61K48/00 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>MOLECULAR AND CELLULAR BIOLOGY, vol. 13,no. 9, September 1993 WASHINGTON DC, USA, pages 5567-5581, M. BLACKETER ET AL. 'Regulation of dimorphism in Saccharomyces cerevisiae: Involvement of the novel protein kinase homolog Elm1p and protein phosphatase 2A.' see abstract</p> <p style="text-align: center;">--- -/--</p>	1-11

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

9 May 1995

Date of mailing of the international search report

19.05.95

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Authorized officer

Nooij, F

INTERNATIONAL SEARCH REPORT

Application No
PCT/US 95/01806

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MOLECULAR AND CELLULAR BIOLOGY, vol. 11,no. 11, November 1991 WASHINGTON DC, USA, pages 5767-5780, A. HEALY ET AL. 'CDC55, a Saccharomyces cerevisiae gene involved in cellular morphogenesis: Identification, characterization, and homology to the B subunit of mammalian type 2A protein phosphatase.' see abstract see figures 5,7 ---	1-14
A	CELL, vol. 72,no. 4, 26 February 1993 CAMBRIDGE MA, USA, pages 621-633, R. MAYER-JAEKEL ET AL. 'The 55 kD regulatory subunit of drosophila protein phosphatase 2A is required for anaphase.' see abstract see discussion see figures 2,3 ---	1-11
A	THE FASEB JOURNAL, vol. 5,no. 4, 11 March 1991 BETHESDA MD, USA, page A832 S. ZOLNIEROWICZ ET AL. 'The B subunit of protein phosphatase 2A is homologous to the S. cerevisiae CDC55 gene product.' see abstract 2685 ---	1-11
P,X	MOLECULAR AND CELLULAR BIOLOGY, vol. 14,no. 5, May 1994 WASHINGTON DC, USA, pages 3350-3363, J. WEINSTEIN ET AL. 'A novel mammalian protein, p55CDC, present in dividing cells is associated with protein kinase activity and has homology to the Saccharomyces cerevisiae cell division cycle proteins Cdc20 and Cdc4.' see the whole document -----	1-19,22, 23

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 95/ 01806

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 15-20 (partially, as far as an in vivo method is concerned) and 21 completely are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

